

The University of Chicago

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IN THE LIMB BUD OF THE FROG

II. SELECTIVITY OF NERVE FIBERS FROM THE
DORSAL AND VENTRAL ROOTS IN THE
DEVELOPMENT OF THE FROG LIMB

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF ZOOLOGY

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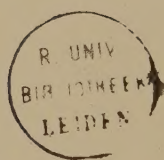
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DEVELOPMENT OF THE INNERVATION PATTERN IN THE LIMB BUD OF THE FROG

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FIFTEEN TEXT FIGURES AND TWO PLATES (TEN FIGURES)

INTRODUCTION

It is surprising that the normal development of nerves in the limb of the frog should have received so little attention, especially as the tadpole has been used repeatedly since the beginning of the century for experimental studies on innervation.

Before undertaking a proposed experimental project, it was found necessary to make this detailed study of the process of nerve pattern formation by examination of its progress at various stages in the ontogeny of larval frogs.

Since the factors determining pattern formation are intrinsic to the limb itself (Harrison, '35), it has been supposed that the limb components (skin, muscles, blood vessels) act somehow, either through chemical attraction, local electrical potentials, or by mechanical guidance, to fashion the invading nerve fibers into the typical pattern. However, no critical evidence could be obtained to show that any type of agent was adequate to direct nerve outgrowth or to produce a pattern.

A renewal of investigation on the problem of normal pattern development seems especially appropriate since recent studies by Weiss ('33, '34, '41) have brought to light some of the laws which govern the oriented outgrowth of nerve fibers in tissue culture and in deplantation experiments. It is of

¹ This research was done under the direction of Dr. Paul Weiss in partial fulfillment of the requirements for the degree of Doctor of Philosophy. It has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

particular interest to determine whether in the normal embryonic processes there is evidence of the action of these factors and whether they seem adequate to account for the establishment of the typical nerve pattern in the limb.

The anuran larvae are especially suited for the purpose of such a study since in these the hind limbs appear much later in ontogeny than in other vertebrates, including the urodeles. As a consequence it is possible to deal with an essentially embryonic limb in the larval form in which the ganglia and cord are relatively far advanced in development, thus permitting of a much more localized and precise operation than can be performed on the much younger stages of urodeles or other vertebrates.

METHODS

The present studies on limb nerve development were carried out on larvae of *Rana pipiens*, which had been reared from eggs in the laboratory under optimal conditions of feeding and temperature. These larvae were carefully staged and then fixed in either Bouin's or Zenker's fluid. Tissues were imbedded in paraffin and sectioned at a thickness of 8 micra. Heidenhain's modification of Mallory's azan triple stain was used for cytological elements, and Bodian's activated protargol-reduced silver impregnation for the neurofibrils.

Besides direct microscopic study of the sections, two- and three-dimensional reconstructions of the nerve pattern were made with the aid of a Bausch and Lomb microprojector. Two-dimensional reconstructions consisted of superimposed drawings of the sections involved. To study the complete limb bud innervation, three-dimensional reconstructions were made by tracing onto 6 by 12 inch glass plates drawings of each section made with the use of the projector. These plates were then assembled in order and cemented together with a plastic (isobutyl methacrylate Polymer) dissolved in xylol. Only the limb bud outlines and the nerve fibers were traced on the plates so that the whole nerve pattern could be seen in relief, permitting a clear visualization of spatial relationships.

Stereoscopic photographs of such reconstructions present the limb and its innervation in their true perspective (figs. 24 and 25).

STAGING

It became desirable in working with frog larvae to be able to refer conveniently to the level in the progress of development which a given animal had attained at a given time. The relation between age, or size, and progress of differentiation fluctuates too much to serve adequately as criterion for staging, especially during the very rapid changes which precede and accompany metamorphosis. A selection was, therefore, made of certain characters correlating better with general development by which the larval period could be divided into a convenient number of stages. For the choice of these characters a study of a statistically significant number of tadpoles has been made and will be published elsewhere. However, for convenience in this paper, outstanding external features by which some of the postembryonic stages may be recognized are listed below. These stages will be designated as "L" (i.e., larval) stages. They follow directly upon the twenty-five stages established by Shumway ('40, '42) for embryos of *Rana pipiens* which will hereinafter be referred to as "E" (i.e., embryonic) stages.

The average body length indicated for each stage is based upon measurement from snout to tip of tail made on larvae kept at a constant temperature of $20 \pm 1^\circ\text{C}$., in individual dishes, and maximally fed upon boiled lettuce.

Photographs showing the limb bud at some of the following stages are given in figures 16-23.

Stage L 1 — Oral suckers have disappeared. Four rows of labial teeth are present. Embryonic pigmentation has been replaced by larval pigmentation in chromatophores. Average body length, 12.5 mm.

Stage L 2 — Length of limb bud equal to one-half of its diameter. Average body length, 16.5 mm.

Stage L 3 — Length of limb bud equal to its greatest diameter. Bud constricted at its base. Average body length, 23 mm.

Stage L 4—Length of limb bud equal to one and one-half times its diameter. No bend in limb bud. Average body length, 34 mm.

Stage L 5—Length of limb bud approximately two times the diameter. Distal half of limb bud bent in ventral direction. No paddle flattening of future foot. Average body length, 40 mm.

Stage L 6—Medio-lateral paddle flattening of distal portion of limb bud. No indication of interdigital notches. Average body length, 43 mm.

Stage L 7—More pronounced paddle flattening. Fifth toe prominence separated from fourth by slight indentation of the paddle margin. Average body length, 50 mm.

Stage L 9—Interdigital notches 5-4, 4-3, and 3-2 present. First independent leg movements. Average body length, 56 mm.

Stage L 12—Margin of the lateral half of the 5-4 webb coincides with a line passing from the tip of first toe to tip of fifth toe. Average body length, 68 mm.

Stage L 18—All toe pads of foot are present. Leg and foot used in swimming. Ventral tail fin in region of cloaca reduced. Skin window for foreleg not yet present. Average body length, 71 mm.

Stage L 20—Forelegs freed from skin covering. Average body length, 41 mm.

DEVELOPMENTAL CHANGES BEARING ON INNERVATION

Stage L 1

Just before stage L 1, the hind limb bud is visible externally with the aid of a dissecting microscope as a very slight elevation in the groove between the lateral body wall and the base of the tail. Histological sections reveal that, at this time, some internal organization is already present. Figure 1 shows a frontal section through the limb bud of a larva in stage L 1. The appearance of the limb bud at this stage agrees with the description given by Filatow ('30, '33). The epidermis forming the dome of the bud swelling is distinctly different in appearance from that of the body wall and tail skin, the nuclei

of the inner layer of cells being larger, more spherical and much more closely packed together. That there is also a chemical difference is indicated by a marked color differentiation in the gold-toned silver impregnations, the epidermis of the limb bud being bluish, while elsewhere over the body it appears in purple or red.

Also noteworthy is the specialization of the somatopleural cells lining the portion of the coelom directly opposite the limb disc (ss, fig. 1). Unlike the extremely flattened squamous

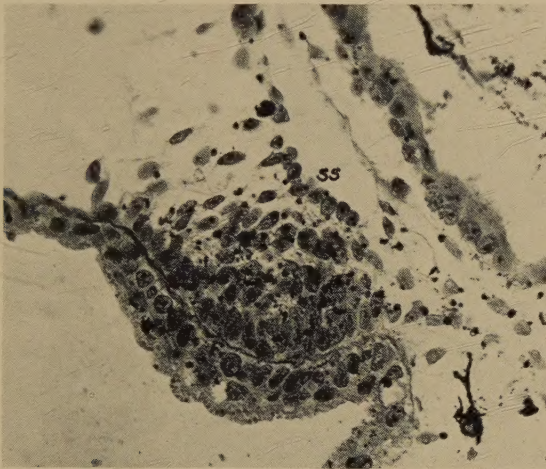


Fig. 1 Frontal section through the limb bud of a stage L1 larva. ss, specialized portion of somatopleure. Mallory's azan triple stain. $\times 420$.

epithelium cells which form the coelomic lining elsewhere, those of this region are rounded and quite closely packed together, having lost their strict alignment. Occupying the space between the epidermis and the somatopleure, there is a mass of mesenchyme cells, densely packed near the epithelium of the bud and already showing a looser arrangement in the region bordering on the somatopleure. This latter looser portion, which becomes more prominent in the two succeeding stages, forms a connection between the limb base and the somatopleure and will be referred to as the "mesenchyme bridge".

The crowding of cells in the somatopleure, the radiating lines of the mesostroma and the elongated shape of the bridge cells all accord with Filatow's contention that the mesenchyme of the limb is being built up by immigration of cells from the somatopleure.

The spinal nerves 8, 9, and 10 approach the bud from a mediodorsal direction and lie between the somatopleure and the myotomes. They consist of two distinct types of fibers, a small number of relatively coarse fibers and a bundle of comparatively faint fibers, so fine as to resemble in size the fibrillae within a large axon. All the fibers of the first category can be traced easily through the serial sections. Some are seen leaving the main trunks at intervals to penetrate between the myotomes and become distributed through the already well differentiated body musculature. Only a very few of these larger fibers reach the level of the tail base where they end in the skin.

The fine fibers, which form a separate bundle lying adjacent to the heavier ones, taper peripherally, becoming finer and fainter until, at the level of the limb bud, they become invisible even under highest magnification.

It must be borne in mind that what appears in silver preparations to be the end of a growing fiber tip need not be considered its actual termination. Beyond the visible end, the fiber often extends in very fine pseudopodia which either fail to be sufficiently impregnated or are below the limit of microscopic visibility.

A few fibers of larger diameter and taking a heavier impregnation enter the epidermis of the limb bud at its median border (not visible in fig. 1), having come from a branch of the eleventh spinal nerve. This is the beginning of an intra-epidermal innervation which will be mentioned later.

Stage L 2

The visible changes occurring during the interval between the first and second larval stages are chiefly those of size and proportion. Accompanying these growth changes, certain positional shifts are noticeable by stage L 2 which materially affect

the development of the limb. To illustrate these shifts, diagrammatic frontal sections through animals at stages E 25 and L 2 are shown in figure 2.

Comparison of the two diagrams makes apparent the following changes: (1) That portion of the mesenchyme anchoring the limb bud to the somatopleure, which has already been designated the bridge (b), has been considerably increased in extent. (2) The axis of the limb bud (a-a) has shifted from

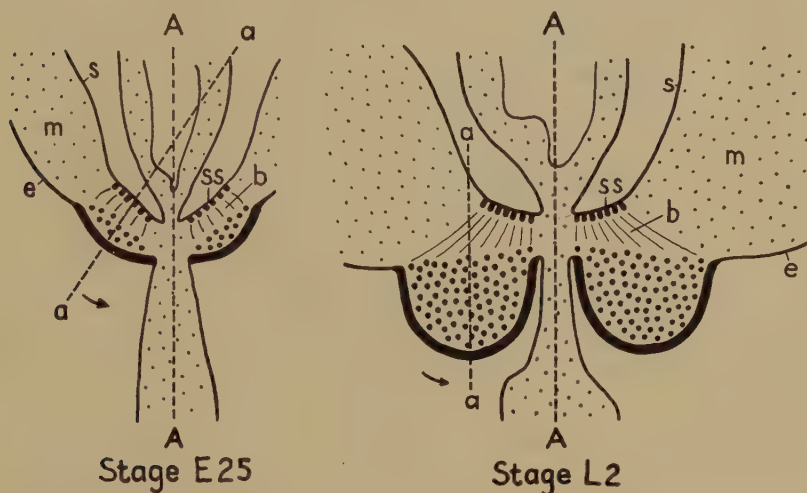


Fig. 2 Diagrammatic frontal sections through the limb buds of larvae in stages E 25 and L 2 to illustrate the positional shift of the bud occurring during this interval. A-A, body axis of the larva. a-a, axis of the limb bud. b, region of the bridge mesenchyme. e, skin. m, subdermal mesenchyme. s, somatopleure lining the coelom. ss, specialized portion of the somatopleure.

its oblique position of the earlier stage to one almost parallel with the body axis (A-A). This shift results in the transposition of the original anterior and posterior margins of the limb disc in stage E 25 to the respective lateral and medial surfaces of the bud in stage L 2. (3) During this shift the distance between the lateral border of the limb bud and the specialized portion of the somatopleura (ss) increases much more than the distance between the latter and the mesial border. In consequence, the bridge mesenchyme, being anchored to both the

limb bud and the somatopleura, shows much greater stretch in its lateral portion than elsewhere.

These changes in the limb bud occur in consequence of developmental happenings in other parts of the body. Filatow ('33) observed that during this interval there is a swelling of the intercellular matrix of the mesenchyme (m) found under the skin, causing an increasingly greater separation of the skin from the somatopleure. A tension is thus exerted upon the stroma of the bridge which extends between these two tissues, accounting in part for its rapid increase in extent. Coincident with this swelling is the establishment of the intestinal spiral, which further distends the body of the larva in front of the limbs and exerts a lateral pull on the limb bud. These forces, together with the rapid increase in diameter of the limb bud disc itself, are responsible for its lateral displacement and the shift of its axis, just described.

The stresses created by these shifts finds striking expression in the fine structure of the mesenchyme bridge at stage L 2 (fig. 3). The elongated shape and parallel arrangement of the nuclei, and the definite orientation of the mesostroma of the bridge (b), indicate clearly the direction of the tensional forces and their relative intensity at different points. That stresses resulting from organ growth may have such moulding effects on the surrounding mesenchyme has been emphasized by Weiss ('33, '39).

The nuclei of the mesenchyme within the bud are not evenly distributed. A layer of more densely packed cells (c) has formed next to the epidermis, which is thickest at its proximal dorsomedian region, opposite the point of entry of the nerves.

Many fine nerve fibers can now be traced into the mesenchyme of the bridge (nf) where they are seen to extend in the direction of the limb bud. After entering the bridge, the fibers of the three contributing spinal nerves have intermingled in such a way that any branches which may later emerge from them could contain fibers from all three of the lumbo-sacral segments. This region of fiber commingling is the future sciatic

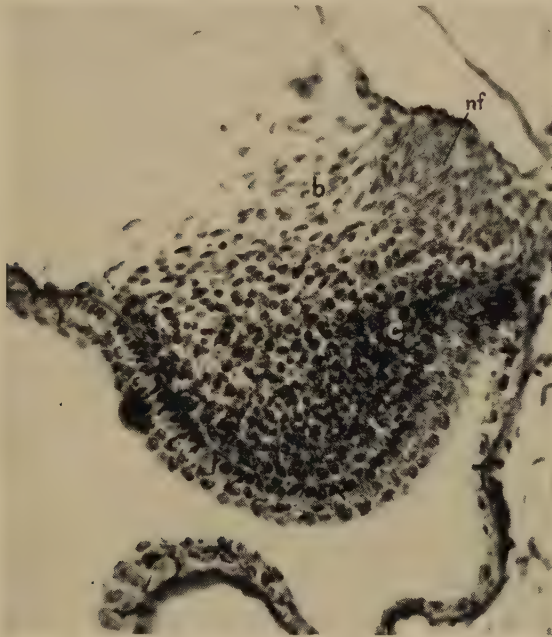


Fig. 3 Frontal section through the limb bud at a stage L 2 larva. Impregnated with silver according to Bodian and counterstained with azan. $\times 200$. b, mesenchyme of the bridge. c, condensation of limb bud mesenchyme. nf, nerve fibers.

plexus. It should be noted that at the time of its formation it is near the level of the base of the limb.

Stage L 3

From the beginning of its development, the limb bud has had a well differentiated epidermis consisting of two cell layers resting on a distinct collagenous basement membrane. At stage L 2, the outer layer has lost its squamous appearance so that now both layers have large spherical nuclei which lie close together. By stage L 3 (fig. 4), the outer layer around the basal part of the bud is flattening somewhat, while over the distal portion, both layers retain a more embryonic appearance.

Further evidence of the anchorage of the limb bud to the somatopleure by the mesenchyme of the bridge is seen here

in the constriction of the bud at its base, so that by stage L 3, it is no longer greatest in diameter at this level.

The distribution of mesenchyme within the bud at stage L 3 is, in general, the same as during the preceding stage. Although, on the whole, the cells are quite evenly distributed, the peripheral condensation seen in stage L 2 is still present,

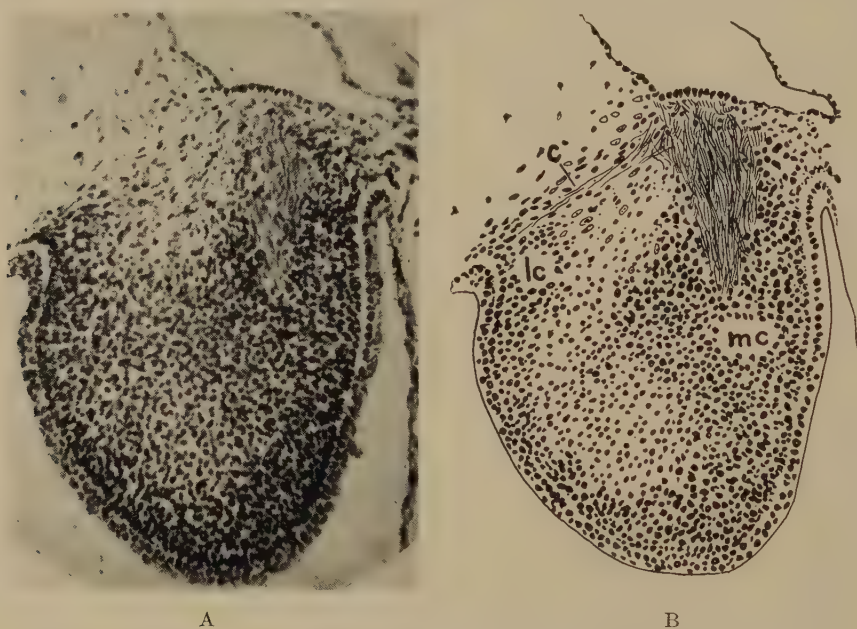


Fig. 4 Frontal section through the limb bud of a stage L 3 larva. A, photomicrograph to show the cellular details. Silver impregnation. $\times 150$. B, composite drawing to indicate extent of development of the innervation pattern. c, cruralis division. lc, lateral condensation of the mesenchyme. mc, median condensation of the mesenchyme.

having increased generally in thickness, especially at the lateral margin of the base and along the whole median surface of the bud. The thickening at the lateral margin extends medially and anteriorly into the bridge mesenchyme, its nuclei being greatly extended in that direction (lc, fig. 4). The median condensation extends from the tip to the base, with its greatest extent in the proximal half, where it reaches to the center of

the bud (mc, fig. 4). Into this more condensed mass of mesenchyme passes the main body of the nerve fibers from the plexus, the portion which represents the later sciatic division.

At this stage the fibers can be traced into the bud for about one-third of its length. Still very fine and taking only a slight impregnation, they are seen to intermingle within the plexus in a sort of feltwork. Yet, throughout the plexus and down into the limb bud, they are grouped into numerous fascicles, slightly separated from each other by open spaces. It is difficult to trace the individuality and continuity of the fascicles because of their number and irregularity and especially since fibers, singly or in small groups, frequently pass from one to the other.

Not all the fibers from the plexus enter the median mesoderm mass. A considerable number are turned laterally in the direction of the outer border of the limb bud, apparently following the stretched stroma in this region. This is the beginning of the cruralis division (c). Although it consists of numerous fibers where it leaves the plexus, this division tapers rapidly, so that only a very few of its fibers can be seen to have actually reached the condensed mesenchyme on the outer surface of the bud.

At stage L 3, no indications of a future innervation pattern are discernible other than this bifurcation of the nerve mass into the main cruralis and sciatic divisions. On the whole, the fibers form a fairly compact mass so that the advancing tip of the sciatic branch has roughly the shape of a cone. On the surface of the cone, and particularly at its tip, individual fibers are frequently seen leaving the mass to penetrate for a short distance between the cells of the mesenchyme.

At this point it should be mentioned that capillaries and small blood vessels are found in the limb bud as early as stage E 25, and are actively permeating the tissue throughout stages L 1 and L 2. By stage L 3, three main vessels, two venous and one arterial, have been established longitudinally through the limb with numerous smaller ones passing between. It is easily observed that the cruralis division of the nerves at this time

has no accompanying blood vessel, and the sciatic nerve, while it courses parallel to the sciatic vein through the same region of mesenchyme, is not applied closely to the vessel.

Stage L 4

While there is only slight change in external appearance of the limb bud during the interval between stages L 3 and L 4, several important developments are histologically apparent (fig. 5). The epidermis still shows a gradation from the embryonic condition at its tip to an almost fully developed larval condition near its base. At the lateral and dorsal regions of its base where differentiation has progressed farthest, the deeper layer has become pseudostratified, so that the nuclei are distributed in three layers.

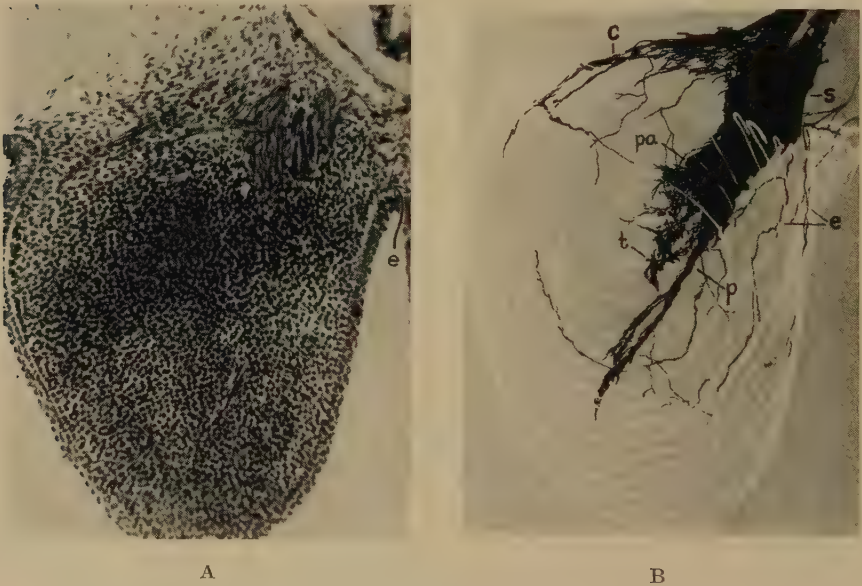


Fig. 5 Limb bud of a stage L 4 larva. A, photomicrograph of an 8-micra section impregnated with silver. $\times 110$. B, photograph of glass plate reconstruction to show extent of development of the innervation pattern. (White lines indicate the outlines of the limb bud). c, cruralis division. e, fibers of the epidermal primary innervation. p, peroneus brach. pa, profundus anterior branch. t, tibialis branch.

Distinct differences in the degree of compactness of the mesodermal nuclei make it possible by this time to identify the anlagen of the femur, and the tibia and fibula, and the larger muscle groups. Of these only the femur anlage gives any suggestion of its definitive shape, the others being merely centers of cellular condensation which can be identified only by their spatial relationships. These all appear to have differentiated from the mesodermal condensation, which in stage L 3 seemed to bud off from the median wall of the peripheral thickening and into which the sciatic nerve branch was then penetrating.

Along with these changes in the mesenchyme occurs a rapid development of the nerve pattern. Soon after stage L 3, the future nerve branches are foreshadowed by a splitting up of the peripheral portions of the nerve mass, accomplished apparently through a grouping of smaller fascicles already observed in the previous stage. A study of the condition of the pattern at stage L 4, in comparison with those of the following two stages, shows that what will be the main mixed trunks of the thigh and shank are already present, i.e., (1) the forking distal to the plexus into the cruralis and sciatic (c and s), (2) the division of the sciatic into the peroneus and tibialis (p and t), (3) the separation of the peroneus into the medial and lateral branches, and (4) the division of the tibialis into the profundus and superficialis (suralis) branches. Besides these, two purely cutaneous branches (*Ramus cutaneous femoris lateralis* and *Ramus cutaneous lateralis*)² can now be traced into tissue immediately subjacent to the epidermal layer.

One motor branch is clearly established. This appears as a tuft of fibers leaving the sciatic division near the point of its bifurcation into tibial and peroneal (pa). It penetrates for a short distance the denser mesenchyme lateral to the nerve mass and will eventually form the large profundus anterior branch to the extensor group of muscles in the thigh. No other purely motor branches are as yet distinguishable, unless one were to consider the sporadic single fibers which leave the

² Anatomical nomenclature according to Ecker und Wiedersheim (1896).

future mixed trunks irregularly along their courses to be the beginnings of muscle innervation.

Mention has been made of the appearance, at an earlier stage, of fibers within the epidermal layer. At this time, they are seen to enter the wall of the bud at its median border through two or three fine branches from a division of the eleventh spinal nerve. These branches (e), containing from one to three large fibers, course backward irregularly through the epidermis, giving off numerous branches. They do not reach to the lateral surface of the limb bud, but form a loose network in the epidermis of the medial surface and in the more rapidly proliferating tip epithelium.

In this stage, where the nerve branches are longer and more slender than in the preceding stage, it is even more evident that the courses of the newly forming nerve twigs and rapidly growing tips are independent of existing or developing blood vessels. For while the larger, more proximal branches of both systems tend to occupy the same tissue spaces, the finer and more distal branches, which represent the actively advancing portions, travel independently and may even cross at right angles.

Stage L 5

The mesodermal primordia in the limb bud of a stage L 5 larva have become much more distinctly outlined, so that it is possible to identify not only the skeletal elements of the thigh and shank, but also the muscle anlagen of the extensors and flexors of the hip and knee joints. Less distinct condensations at the distal end represent already the elements of the foot. Correlated with the condensation of mesenchyme in these primordia is the loose appearance of the tissue between them. It is within these spaces that the larger blood vessels and the nerves are now found to course.

The reconstruction of the nerve pattern of this stage (fig. 24) shows clearly that nearly all the branches of the adult leg have, by this time, been established. All of the cutaneous branches can be identified, and all of them traced into the

dermis. Three other branches (shown to be sensory by experiments to be published elsewhere), which in older stages can be traced into the ligaments and tendons of the knee and ankle joints, are also well established. This is rather surprising since, even in the adult frog, these branches are so small that only one of them has so far been found described in the literature. This latter is the *Ramus articularis genu et pedis*, mentioned by Ecker (1896). It leaves the peroneus near the knee joint. The other two of these, presumably proprioceptive nerves, run to the knee, one from the peroneus, which will supply the antero-lateral surface of the joint, and the other from the *tibialis profundus* to its median region. These might be designated the *Rami articulares genu lateralis* and *medialis*, respectively.

Most of the motor branches are also represented. The larger ones are short projections from the mixed trunks, which taper rapidly to a point as they penetrate into the muscle anlagen, while the smaller ones consist of a few frayed-out fibers, which leave the mixed branches along their courses. These branches are more difficult to identify because of their relative shortness and the fact that the muscles which they supply are not clearly segregated. On the whole, the motor branches seem less well established than those to the skin.

The few epidermal fibers derived from the eleventh spinal nerve are distributed along the medial surface of the limb bud (e, fig. 24). None of them extend to the skin of the lateral surface.

Stage L 6

In the tadpole of stage L 6, when externally there appear first indications of a foot paddle, all motor and sensory branches are distinctly identifiable (fig. 25). Furthermore, the increase in the limb bud length, which has occurred since stage L 5, has resulted in the beginning of a process of drawing out of the nerves to assume more nearly their final proportions.

Most of the cutaneous branches have not only reached the skin, but have coursed along its inner surface, giving off collateral branches at intervals. The extent of the epidermal nerves from the eleventh spinal segment is very similar to that in stage L 5. These fibers persist at least until late larval life, becoming progressively less conspicuous. By the time the toes are fully formed, they consist of no more than two or three single fibers on the median surface of the leg, branching only in the foot and toes. Apparently they are wholly independent of the leg innervation supplied through the sciatic plexus. There appears to be no functional significance attached to this dual source of innervation since tests on tadpoles of various ages showed no difference in response to stimulation of the medial and lateral surfaces. Metamorphosed frogs have not been examined to determine whether this innervation is retained in the adult.

Along with the lengthening of the leg, its muscle primordia are also extended longitudinally. Although origins and insertions have not yet differentiated, the myoblasts of the future muscle belly have assumed spindle shape and, particularly in the region of the thigh, cross striations are beginning to appear. Correlated with these muscle developments, there has been a lengthening of the nerve branches innervating them. The forking of the larger motor branches, which in stage L 5 had barely begun, is here well established. Furthermore, the tips of many motor fibers are associated with the newly striated muscle fibers in a fashion quite typical of mature amphibian motor endings. It might be pointed out here that no movement of the limb occurs until three stages later (stage L 9).

As far as concerns the innervation of the limb, with the possible exception of the foot, the pattern foundation is essentially complete by stage L 6. It is, therefore, unnecessary to describe here the later stages, in which the systems already laid down are merely filled out and extended.

THE SOURCE OF LIMB INNERVATION

We are not here concerned with the time or manner in which the spinal nerves first make their appearance, since this occurs comparatively early in embryonic life, long before the limb primordia appear. What does interest us is the manner in which the limb bud, situated as it is on the ventral surface and developing at a relatively late larval period, will be furnished with innervation from neurones located in the spinal cord and ganglia of the lumbar region. At an early stage the direction and course of spinal nerves 8, 9, and 10, which are destined to innervate the leg, are not, in general, different from nerves of most other segments. That is to say, they run posteriorly and ventrally between the notochord and myotomes, then laterally between the somatopleure and body wall mesenchyme. The convergence of the eighth, ninth, and tenth nerves to the site of the future limb would appear to be a natural result of the architecture of that region, since the coelomic linings of the right and left body walls themselves converge and unite at the region of the cloaca. Such an interpretation would be in line with the emphasis on the role played by the mechanical configuration of embryonic organs and tissues in the orientation of nerve outgrowth found in the writings of His (1887) and Harrison ('10 and '14) and more recently elaborated by Weiss ('34, '41). In this way the appropriate spinal nerves are already brought into the vicinity of the future limb bud before the appearance of that organ. The active proliferation within the tissue of the limb bud anlage, after it first appears in stages E 25 and L 1, is then able, by means of chemical and mechanical actions to effect micellar orientation within the surrounding stroma in such a way as to act as a passive local collector of all growing fiber tips within its reach (Weiss, '34).

Reference again to the figures of early stages will show that the fibrillae of the nerve mass growing into the mesenchyme bridge have an orientation that coincides with the arrangement of the background of cell strands; that the advancing fiber tips follow the configuration of the mesostroma can clearly

be seen in the illustration of stage L 3 (fig. 4), particularly in the region of the newly appearing cruralis division.

It has already been stated that limb buds of anurans appear relatively much later in ontogeny than in other amphibians. In the frog embryo, a primary innervation for the trunk musculature and skin is established and is functional long before the appearance of hind limb primordia. It is the fibers of this primary somatic innervation, extending from the cord to the region surrounding the future limb, which then serve as a conducting pathway for the later fiber outgrowth in a manner which Weiss ('41) has called "growth by application". The latter secondary innervation becomes visible in silver preparations soon after the appearance of limb primordia and is destined to supply the limbs.

The fibers thus furnished to the early limb bud consist of axons of both sensory and motor neurones. This was determined by tracing the fibers back to both the dorsal and ventral roots, as described below, and by histological studies of tadpoles in which the fibers coming from the cord had been allowed to degenerate after unilateral section of the ventral roots supplying the sciatic plexus. Such animals showed that the volume of fibers at the level of the limb bud was smaller on the operated side than on the control side, a condition which would be true only if motor as well as sensory fibers had originally been present.

It has been noted that all the fibers entering the limb bud through the sciatic plexus are very fine and take a light silver impregnation. The contrast in size between these and the fibers which supply the adjacent body wall and musculature is very marked when they appear side by side in the mixed nerves above the plexus. A cross section of the ninth spinal nerve of a larva in stage L 2 is shown in figure 9, from which it is seen that the fiber diameters may differ by several hundred per cent. By means of this difference, it is possible to trace the limb bud innervation back to its sources in the ganglia and cord. With growth of the larva, there is progressive increase in diameter of fine fibers. The most rapid change occurs

between stages L 1 and L 4, followed by a comparatively gradual increase between L 4 and L 16. On the other hand, there is a more steady augmentation of the number of these fibers up to at least stage L 16 (fig. 13).

These changes in size and number of spinal nerve fibers are intimately related to changes taking place in the ganglia. At the beginning of stage L 1, the spinal ganglia of nerves 8, 9, and 10 contain comparatively few cells. About twenty-five to thirty of these are large and already possess large nuclei and distinctly visible processes. They make up the bulk of the ganglion; the remaining cells are smaller and stain more darkly in azan. By stage L 3 there has been a considerable increase in the number of the smaller cells while the larger ones, which are isolated in a lateral cluster, still number approximately thirty or less. The increase of the small cells continues steadily. By actual count, in the ninth ganglion of a stage L 6 tadpole, there were twenty-three large and 2,190 small cells (fig. 10). That many of the small cells are real ganglion cells is already evident before stage L 6 since fibers can, by this time, be seen to originate from them. Clearly these are the cell bodies of some of the fine fibers found in the spinal nerve, while the heavy fibers are processes of the large ganglion cells. A similar set of large and small fibers extend from the ganglion up the dorsal root to the sensory column of the cord. The number of large fibers in this root is the same as the number of large cells in the ganglion and remains fairly constant in all early larval stages, while the number of small fibers increases with the number of small cells in the ganglion.

The ventral roots are likewise composed of two sizes of fibers comparable to those in the dorsal roots. The large fibers come from the region of the motor neurones lying in the ventral gray matter and leave the cord just lateral to the median ventral fissure. The fine fibers leave the cord by several small branches at points more dorsal and lateral. They unite and join the bundle of larger fibers in their course toward the ganglion. The number of heavy fibers in a mixed nerve trunk is equal to the sum of those in its dorsal and ventral roots.

Figure 14 shows a diagrammatic representation of the relationship of these fibers to their cell bodies and end organs.

The description of spinal nerves and their ganglia given above holds only for the lumbar and brachial regions. Cross sections of spinal nerves 5, 6, 7, and 12 differ markedly from those of the limb segments in the comparative scarcity of fine fibers (figs. 7-9). The ganglia of these segments contain correspondingly few small cells (fig. 6).

In studying the ventral roots of the spinal nerves of young amblystoma larvae, Youngstrom ('40) finds a new system of fine motor fibers appearing among the older fibers of the primary innervation of the embryo. Because this secondary innervation appears at the time of the development of independent withdrawal reflexes in the limb, he considers it the mechanism for the individuation of localized movements from an earlier more generalized behavior pattern. In the larva of *Rana pipiens*, however, where the nerves have been followed to their peripheral endings, heavy fibers can be traced to only those end-organs which are functionally innervated during the embryonic period, namely, somites and skin, while the newly developing larval limbs are exclusively supplied by the

Figs. 6-13 Spinal ganglia and nerves from larvae of various stages to show the size difference between the cell bodies and fibers of the primary and secondary innervation. Silver impregnation according to Bodian.

Fig. 6 Sixth ganglion of a stage L 2 larva. (Does not furnish fibers to limbs.) Note relatively small number of small secondary cells. $\times 340$.

Fig. 7 Cross section of spinal nerve of same segment showing very few fine fibers. $\times 340$.

Fig. 8 Ninth ganglion of stage L 12 larva. (Furnishes fibers to hind limb.) Note comparatively large number of small secondary cells. $\times 340$.

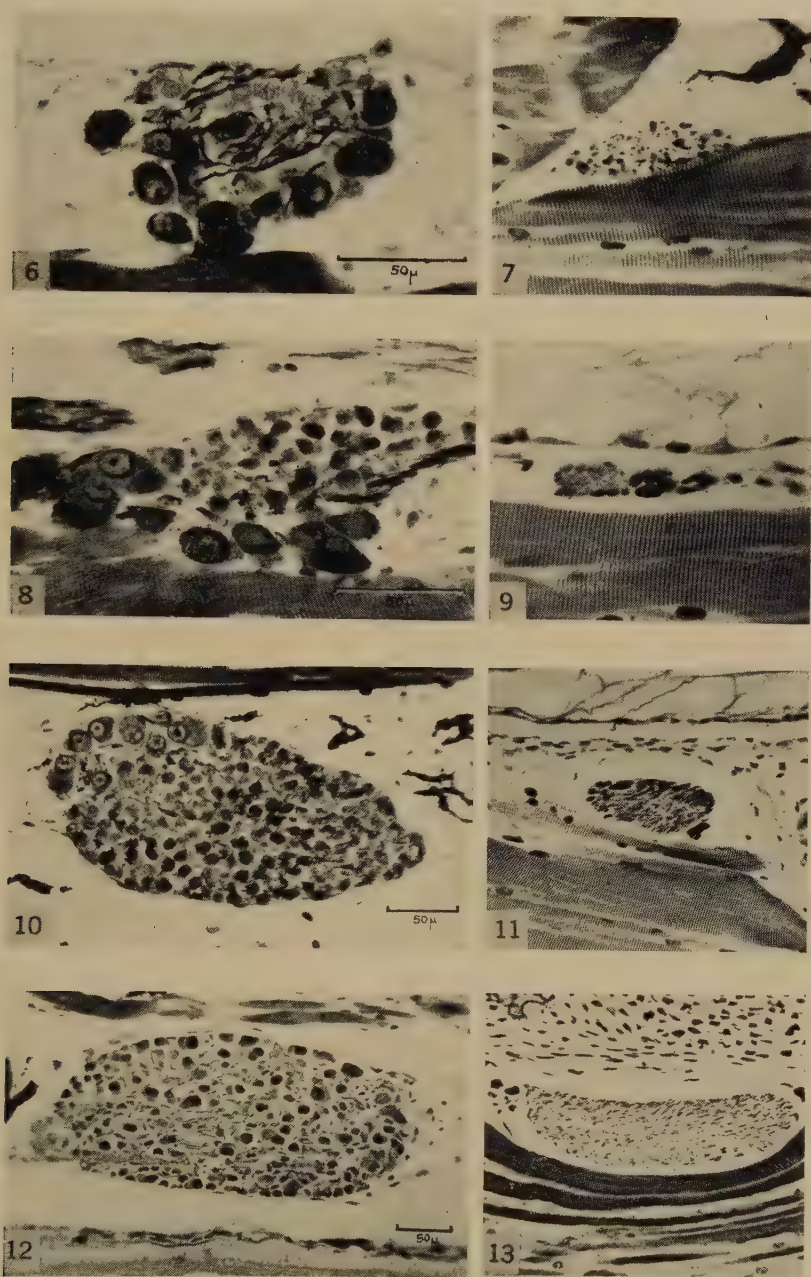
Fig. 9 Cross section of spinal nerve of same segment. Note the mass of very fine fibers beside the few large ones. $\times 340$.

Fig. 10 Ninth ganglion of a stage L 6 larva. Contrast the number of secondary ganglion cells with the cluster of comparatively large primary cells at the left. $\times 190$.

Fig. 11 Cross section of spinal nerve from same segment. Note the increase in size of the secondary fibers over those of stage L 2. $\times 190$.

Fig. 12 Ninth ganglion of a stage L 16 larva. Primary cells not easily distinguishable from the secondary. $\times 150$.

Fig. 13 Cross section of spinal nerve of same segment. Note small number of large primary fibers still present. (At left.) $\times 150$.



Figures 6 to 13

fine fibers of the secondary innervation. Since in the frog limb there are no primary motor fibers prior to the establishment of the definitive innervation, there seems to be no anatomical evidence for the assumption that localized behavior patterns of the limb are preceded by a generalized total pattern of true limb movements mediated through a special nervous mechanism.

An attempt to correlate the development changes in the limb bud with those taking place in their source of innervation leads to the following conclusions. Prior to the appearance of the hind limb primordium, a neural pathway has been in existence, extending from the source of fibers to the very

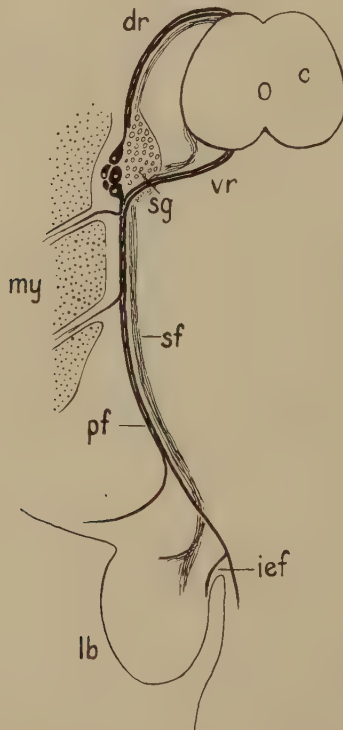


Fig. 14 Schematic drawing to illustrate the relationship of the primary and secondary fibers to the cord, ganglia and end-organs. c, spinal cord. dr, dorsal root. ief, intra-epidermal primary fibers. lb, limb bud. my, myotomes. pf, large primary fibers. sf, fine secondary fibers. sg, spinal ganglion. vr, ventral root.

“door-step” of the future limb. The nerve fibers which form this “pathway” are part of a primary embryonic innervation which has established early end connections with adjacent trunk muscles and skin, but will never enter the leg. At about the same time that limb bud anlagen are to appear, a mass of new cells begin to differentiate in the ganglion, and their axons, along with similar fine fibers originating from the cord, following the “pathway” by application, are conducted toward the base of the new limb.

NERVE PATTERN FORMATION

The definitive nerve pattern of the frog limb consists of many divisions, trunks, and branches. In its development, each of these is established at a different time in different parts of the limb bud, and hence, under varying local conditions. Each part encounters different obstacles and is subjected to differential stretches and shifts as the elements of the limb increase in size and alter their relative positions. The process of pattern formation consequently must be approached through a consideration of the factors operating in the establishment of each of its branches separately. In analyzing the mechanics of nerve growth and pattern formation, Weiss ('41) summarizes the process of establishment of an embryonic nerve in the following words:

“Thus the establishment of a peripheral nerve involves three overlapping phases: First, the free outgrowth of a group of pioneering fibers through non-nervous surroundings; second, the bound outgrowth of subsequent fiber generations along the line laid down by the pioneering fibers; and third, the towing process in which the nerve is drawn out by the growth and dislocations of its terminal tissues.”

If the forces determining the end attachment of nerves act only on their pioneer fibers, the agencies responsible for the basic configuration of nerve pattern in the limb must be active at a time preceding the building up, or filling out of its branches. From the description of limb development here presented, this would necessarily be at a very early stage.

Is it quite obvious, then, that pattern development cannot be thought of as an invasion by nerves of a visibly differentiated limb, in which the configuration of the nerves is determined by the spatial arrangement of limb elements already present, e.g., blood vessels, muscles or skin. Such a conception seems to be held by some who, like Hamburger ('28), have drawn inferences mainly from terminal stages. One should, rather, think of the visible differentiation of limb elements as occurring within mesenchyme which has previously been invaded by pioneer nerve fibers and in which nerve branches are already forming.

It remains then to discover the forces which may operate to establish the courses of the pioneer fibers, and the factors which will produce from these the nerves of the final pattern.

Various agencies have been proposed by different authors to account for the apparently directed outgrowth of nerves. Most prominent among these are the theories of Galvanotropism (Kappers, '17; Child, '21), Chemotropism (Cajal, 1893, '08; Tello, '23) and contact guidance (His, 1887; Harrison, '10, '14; Weiss, '33, '34). Reviews evaluating the different theories in the light of more recent experimental work are presented by Harrison ('35), Detwiler ('36), and Weiss ('41). They agree that all the direct experimental evidence at hand upholds a contact theory of orientation (Young, '42). Studies on the behavior of living nerve fibers carried out by Weiss in tissue culture ('34) and in living animals ('41) have revealed some of these factors which are capable of directing the free outgrowth of the pioneering fibers. In general, they consist of interfaces in the enviroing tissue which may serve as solid substrata for the amoeboid progression of the fiber tips. Such interfacial orientation may be brought about by a variety of vectorial forces, such as mechanical stretch, dehydration and other colloid chemical changes.

It was indicated in the description of stage L 1 tadpoles that even before the visible ends of the ingrowing nerve mass enter the mesenchyme of the limb bud, there exists a very definite structural organization of the mesostroma, probably

related to the course of migration of the mesenchyme cells. During the interval between stage L 1 and L 3, shifts of the limb bud result in mechanical stresses which intensify and alter the configuration of the ground structure, particularly in the region of the mesenchyme bridge. That these definitely oriented finer structural elements are related to the subsequent course of nerve fibers which soon enter and pass through the bridge, is suggested by the congruity of their patterns. The alignment of the stroma strands is much more clear cut along the medial and lateral borders of the bridge than in its interior. The appearance within these regions of the two main divisions which emerge from the sciatic plexus is a notable instance of a definite relationship between the pattern of the preneuronal substratum and the developing pattern of the ingrowing nerve fibers.

Although less strikingly apparent and not yet adequately studied, local variations can be seen in the arrangement of mesostroma within the limb bud proper, which may be, in part, responsible for the course of the nerve branches to develop later in the region peripheral to the bridge.

Because the fate of the future nerve branches and, hence, of the whole pattern, rests in large part upon the course taken, and the end attachment made, by the tips of the pioneering fibers, the importance of a clear understanding of their characteristics and behavior is evident. Yet definite knowledge concerning the character of fiber terminations is very meager, due largely to the inability to trace them under the microscope to their actual endings.

Boeke ('30, '37), Stöhr ('35) and Nonidez ('37) have studied the terminations of sympathetic and somatic fibers in fixed and stained preparations. Among these workers there is disagreement concerning the structure of nerve fiber endings. The complete individuality of each fiber implied in the neurone theory is questioned by Boeke and Stöhr, who describe terminal anastomosing in the peripheral sympathetic plexus. Boeke ('37) has also found in the early phase of peripheral nerve regeneration an apparent syncytial anastomosing of the

fibers at their terminations. Later, from this primordial plexus, the definite nerve fibers are individualized.

The possibility that such a condition may also exist in embryonic organs at a time when they are first invaded by nerve outgrowths must be conceded. Certain observations on the silver impregnations of early tadpole limbs would at least bear such an interpretation. In stages L 2, L 3, and L 4 faint depositions of silver are visible along extremely fine strands of a fibrous nature, which interlace and anastomose between the mesenchyme cells and which appear to be continuous with the early nerve fibers. Connective tissue within the limb primordium has not yet differentiated its fibers, and the mucoid strands, which are present, have a different appearance from these argentophil fibrillae. There is thus a suggestion that at a very early period in the innervation of the limb, the invading nerves form a reticulum of exceedingly fine neuroplasmic strands within the undifferentiated mesenchyme from which may be individualized the larger discrete fibers following the chemical differentiation of the mesenchyme.

The dearth of knowledge concerning the character of nerve fiber terminations makes it impossible to form a clear picture of the manner or time of their connection with cells of future end-organs. It does seem certain, however, that the actual contact between a fiber and its embryonic end-organ cells is accomplished before there is microscopically visible evidence of this connection.

Although we are unable to determine the exact moment when a pioneer fiber is first connected with its end-organ cells, certain changes soon follow which indicate that such a connection has already been made. It has been suggested by Weiss ('41) that the establishment of its end connection changes the character of a fiber in such a way as to induce other similar fibers to grow out along its surface by "selective fasciculation". The importance of this process in the building up and filling out of the nerve branch has already been emphasized. Thus, the appearance of a fiber cable in the place of independent fibers

would indicate that terminal attachment of the pioneer fiber has been made.

It is possible to tell approximately the stage in larval limb development when the major branches have become certainly established. By stage L 5, every branch of the thigh and shank is present. This may also be true of the foot. In stage L 4, the main divisions and many of their branches are distinctly visible, while probably some of the smaller collateral branches are not yet established. At stage L 3, only the representatives of the large cruralis and sciatic trunks are visible. There are no divisions of the sciatic trunk. From the cruralis trunk, however, a few fibers can be seen to leave at the relative position of the future muscle branch. It is apparent in this case that the cutaneous branches of the trunk precede the muscle branches in their establishment. The relative size of the two types of branches in later stages would agree with this. In stage L 2, no signs of any division of the nerve mass can be seen.

The conditions found at these graded stages would indicate a progressive establishment of nerve branches, beginning at the basal portion in stage L 3 and proceeding distally to completion of the pattern in stage L 5. If then the appearance of these branches indicates the previous anchoring of their pioneer fibers, this process must have occurred prior to stage L 3 for at least the two major divisions. This would be long before there is any visible differentiation of the mesenchyme.

The divisions and branches as established at this early period do not resemble, in their spatial relationships, the mature innervation pattern of the limb. The process by which this preformed nerve pattern is unfolded, or, more correctly, drawn out, can only be understood in terms of the extensive changes in size and shifts in relative position of the muscle and skeletal elements accompanying the growth of the leg. When first formed, the trunks and divisions are short and their branches leave them near their bases. Such a condition results from the fact that the primordia of the future limb elements are arranged side by side in the shallow limb bud. This

telescoped condition of the nerve pattern is immediately apparent from examination of the reconstruction of as late a stage as L 5 although it is more striking in stage L 4. Even in stage L 6, it is seen that the sciatic plexus is close to the limb bud base and that the cruralis division leaves the sciatic at this level. Recalling the condition in the metamorphosed frog, it is apparent that the relative distance between the plexus and the limb has been greatly increased, and that the cruralis has been routed a considerable distance around, dorsal to the iliac bone, before entering the limb proper. In fact, the precartilage of the acetabulum forms within the limb bud (stages L 5 and L 6) and then is left behind by the migration of the whole limb.

Through the lengthening of the leg by muscle shifts, differential growth and the consequent serial alignment of its component elements, the nerve branches anchored to them are towed out to form the main longitudinal trunks with the branches distributed along their length. The effect of this stretching out of the nerve pattern during growth of the limb is diagrammed in figure 15. In this process certain distortions may occur. If the basal portion of a branch comes to lie parallel to the mixed nerve from which it forks, it may later be bound together with the larger nerve by connective tissue wrappings, and thus its point of divergence is shifted distally. On the other hand, an obstacle becoming caught in the fork may separate the fibers of the two branches, causing them to diverge at a relatively more proximal point.

It is obvious that the factors involved in this last phase of pattern formation are those of the mechanics of differential growth of the non nervous elements. As such they have little to do with the determination of the original basic pattern, their effects being expressed only in the arrangement of the nerve bundles trailing behind the growing muscles and skin, and in packing them, together with the blood vessels, into the spaces between the other limb elements.

Our observations of the mutual independence of the courses of blood vessels and nerves during the critical stages in which the nerve pattern is laid down, seem to deprive all claims that

blood vessels are primary agents of nerve patterns of their factual foundations.

This paper, being primarily descriptive, has touched on only a few of many unsolved problems relative to the development of the innervation pattern. Some of these will be discussed in the experimental part of this work to be published later.

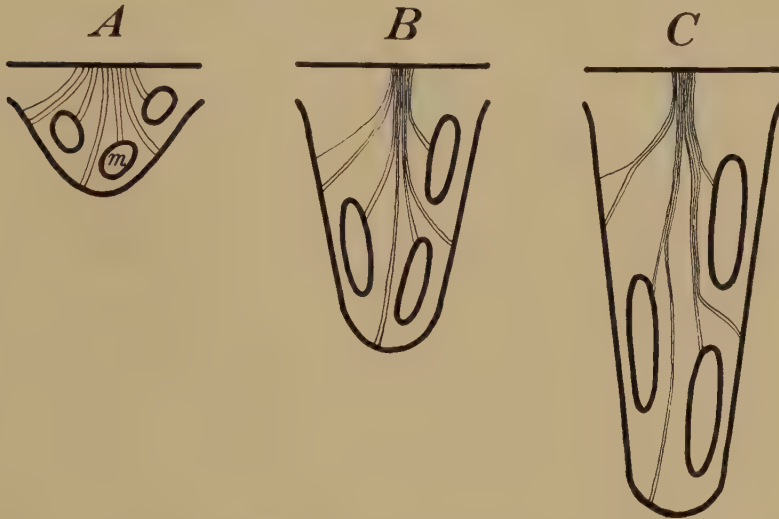


Fig. 15 Diagram to illustrate the effect upon the innervation pattern of the growth and accompanying positional shifts of limb parts. A, nerve pattern when first established at a very early stage. B, intermediate stage. C, late stage with the nerve trunks, divisions, and branches in their definitive positions. m, muscle anlage.

SUMMARY

Since no detailed study of the development of limb nerves in the frog larva has previously been made, it is here undertaken as a preliminary investigation for experimental work to be reported elsewhere.

A study of serially sectioned limb buds of larvae at different stages showed that the establishment of nerve pattern occurs very early, commencing before stage L 3, when as yet there is no tissue differentiation in the mesenchyme of the limb bud, and being essentially completed by stage L 5.

From a consideration of the process of nerve outgrowth in the light of recent experimental work, it is concluded that:

1. The determination of the various branches in the innervation pattern of the limb occurs prior to the time of visible appearance of the branches.

2. The factors which direct the outgrowth of the early fibers and determine the branches of the nerve pattern are to be found in the preexisting pattern in the mesostroma.

3. The possibility of the presence of a neuroplasmic reticulum within the early limb bud mesenchyme, giving rise later to the individualized nerve fibers, is suggested.

4. The determination and establishment of the innervation pattern is not simultaneous for the whole system, but progresses, in general, from the base to the tip.

5. The pattern thus determined is then altered and shaped by the mechanical forces accompanying the differential growth of the parts of the limb.

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PLATE 1

EXPLANATION OF FIGURES

16-23 Unretouched photographs of the tadpole limb at different stages of development. $\times 22$.

16 Stage L 2. The length of limb bud is equal to one-half its diameter.

17 Stage L 3. The length of limb bud is equal to its diameter.

18 Stage L 4. The length of the limb bud is one and one-half times its diameter.

19 Stage L 5. The length of the limb bud is equal to two times its diameter. A slight ventral bend of the distal half of the bud appears. There is no foot paddle flattening.

20 Stage L 6. A flattening of the distal end of the bud is noticeable. No interdigital notches yet present.

21 Stage L 7. Slight indentation between the fourth and fifth toe prominence (at point indicated by arrow).

22 Stage L 9. Interdigital notches 2-3, 3-4, and 4-5 present.

23 Stage L 12.

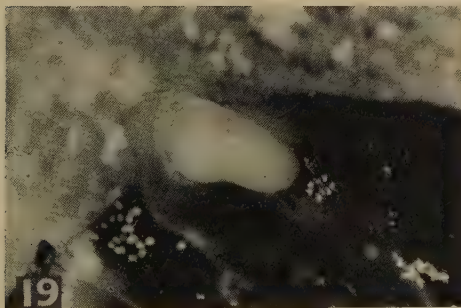
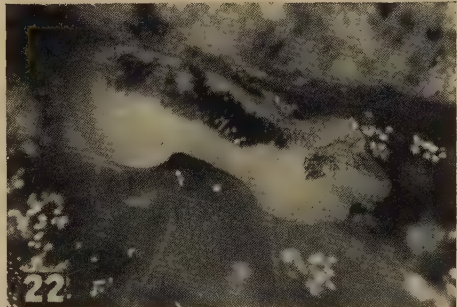
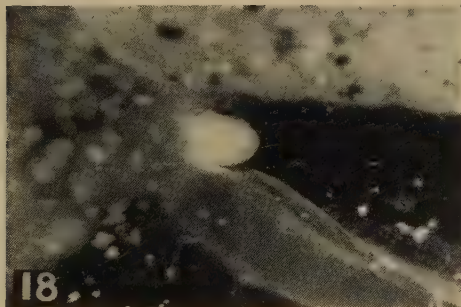
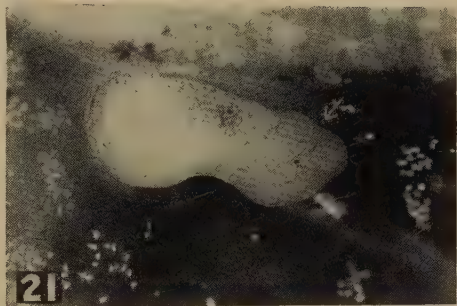
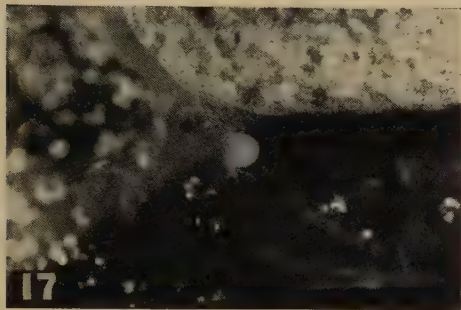
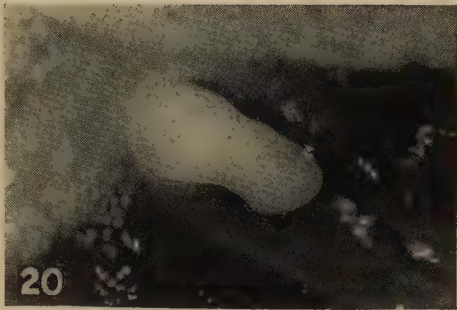
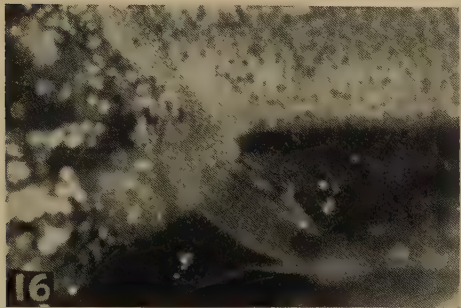


PLATE 2

EXPLANATION OF FIGURES

- 24 Stereoscopic photographs of glass plate reconstruction of a stage L 5 limb bud. (White lines represent limb bud outlines.) e, intra-epidermal primary fibers.
- 25 Stereoscopic photographs of glass plate reconstruction of a stage L 6 limb bud.



SELECTIVITY OF NERVE FIBERS FROM THE DORSAL AND VENTRAL ROOTS IN THE DEVELOPMENT OF THE FROG LIMB¹

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EIGHT FIGURES

INTRODUCTION

In the course of development of the vertebrate embryo, processes form from the neuroblasts of the cord and spinal ganglia which extend into the body tissues and eventually establish terminal connections with effector and receptor organ rudiments. It is apparent that some sort of selectivity must exist to account for the connection of fibers originating in the cord with future muscle fibers and of fibers coming from spinal ganglion cells with the presumptive cutaneous and proprioceptive sense organs. Despite the lack of factual information it has been assumed that an attractive influence is exerted by the terminal tissue upon outgrowing nerve fibers. Cajal (1892) and Tello ('23), among others, agree that this force is presumably chemical in nature. Since, however, it is becoming increasingly apparent that contact guidance, and not chemical attraction from a distance, directs the outgrowing fiber tips (Weiss, '33, '34a, '41b; Weiss and Taylor, '44), this assumption seems to be invalid.

The following experiments were designed to obtain more specific information on the relationship during development of the motor and sensory components of limb innervation to the end organs, and the reaction of one component toward the other in the production of the final pattern. An analysis of these relationships is attempted through experimental isolation of the components. By this method it is possible to study the nerve pattern produced by one component in the absence of the other and to compare these with the normal condition.

Experiments of a similar nature were carried out by Hamburger ('28, '29). In these the operations were performed at an early embryonic stage so that the extent of the injury could not be controlled.

¹ This research was done under the direction of Dr. Paul Weiss in partial fulfillment of the requirements for the degree of Doctor of Philosophy. It has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Furthermore, in some cases tissue disturbances were introduced between the limb and its nerve source. Because of this lack of control over the operation and because no specific nerve stain was used, his results, though suggestive, remained inconclusive with regard to the question of nerve selectivity.

For the purpose of the present investigation the larva of the frog is particularly well suited since in this form the hind limb begins its development relatively late in ontogeny. What in other forms is the embryonic process of limb formation, occurs in the tadpole during the larval stages. This fact permits the removal of the sources furnishing sensory and motor fibers to the limb at a time when the ganglia and spinal cord are well differentiated, yet prior to the establishment of the innervation pattern in the limb. By the technique used the source of either component can be removed alone without injury or disturbance to the other, or to the tissue between it and the limb bud.

An understanding of the normal development of the limb nerves is fundamental for correct interpretation of the results in experimental animals. Accordingly, a careful study of the development of innervation in the normal frog limb was made and has previously been reported (Taylor, '43).

METHODS

Throughout this paper the following terminology will be used. Fibers which reach the limb from the spinal ganglia, are called dorsal root fibers, or D-fibers; while those supplied through the ventral roots are called V-fibers. Limbs of larvae which have had segments of cord removed before the establishment of the innervation pattern in the limb will be designated as V-less (i.e., ventral root less) limbs. Similarly, limbs of larvae in which ganglia have been removed at an early stage are referred to as D-less (i.e., dorsal root less) limbs. These symbols have the same connotation as the terms "anafferent" and "anafferent" proposed by Yntema ('43) which have not been adopted here because of their etymological incongruity. The larval stages² referred to herein have been briefly described in an earlier paper (Taylor, '43).

The animals used for the experiments were *Rana pipiens* larvae most of which had been reared from eggs in the laboratory. The operations were carried out as described below.

Larvae were anesthetized in a 2% solution of ethyl urethane, and transferred for operation into amphibian Ringer's solution.

² The staging of *Rana pipiens* larvae has been proposed jointly by Dr. Kolros and the author. A complete report is being prepared.

For the ventral root extirpation series a longitudinal incision was made into the spinal canal over the lumbo-sacral region, and a section of the cord which included at least the spinal segments 8, 9, 10, and 11 was removed. In some cases the cord was cut at the seventh segment and then pulled out from the posterior part of the spinal canal. The latter procedure resulted in no more serious effects to the animal than the former. The drastic operation of removing part of the cord was finally resorted to after efforts to prevent regeneration of V-fibers into the limb bud by repeated cutting of the ventral roots of the spinal nerves had failed.

In a similar manner the spinal canal was laid open for removal of the ganglia. These were then carefully freed and excised without injury to the ventral roots. In animals at stage L4 or older, this is a comparatively simple operation, for the ganglia have already become compact and are ensheathed in connective tissue. But in tadpoles younger than stage L3 it is difficult to make certain of complete removal of the ganglia which at this time consist of only a few loosely adhering cells. The operation is further complicated in very young larvae by the fact that the V-fibers pass through the ganglionic mass. For these reasons most of the operations on animals of stages L1 and L2 proved later to be incomplete.

Following the operation the spinal canal was closed and the cut edges of the skin were held together until they adhered, and the animal was returned to well water. Operated animals were kept in individual finger bowls until after the limbs had reached the stage of spontaneous function (L12-L14). At this time they were carefully tested for motor and sensory deficiencies by applying tactile stimulation with a hair or steel needle, or by pinching with forceps. After recording reactions, the animals were killed and fixed in Bouin's fluid, embedded in paraffin and serially sectioned at a thickness of 8μ . The mounted sections were then impregnated with silver according to the method of Bodian.

The nerve distribution in the limbs was studied mostly by following the serial sections and recording the nerve courses free-hand. In some cases, however, the nerve patterns were reconstructed by superimposing tracings of the serial sections made with the aid of the micro-projector.

The pattern of innervation of all experimental animals was compared with that of normal animals similarly fixed and impregnated, as well as with the anatomical descriptions given by Ecker (1889) and Ecker and Wiedersheim (1896). To facilitate the comparison of nerve patterns in experimental and normal animals, a diagrammatic representa-

tion of the course of nerves in the normal tadpole limb is given in figure 1. The nomenclature used is that of Ecker.

Two small branches (r. articularis genu lateralis, and r. articularis genu medialis, E and F fig. 1), which could not be found mentioned in the literature, extend to the tendons and connective tissue of the knee, as does also the ramus articularis genu et pedis (G fig. 1). Since electrical stimulation of these nerves did not elicit any muscular contraction, and in view of their anatomical relationships as well as their

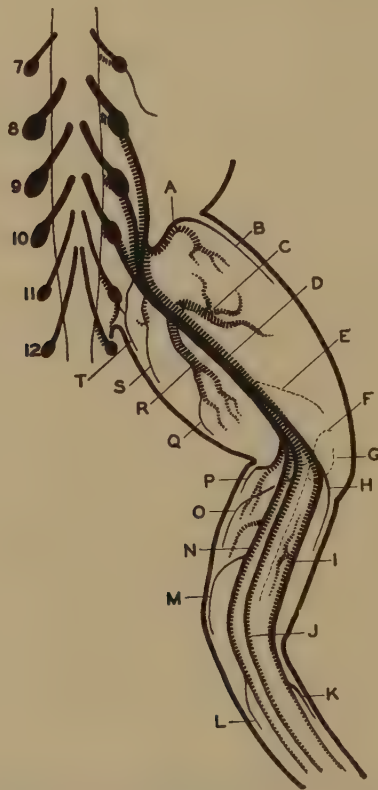


Fig. 1 Diagram of innervation of a normal tadpole limb. Dorsal root component shown in solid lines. Ventral root component shown in discontinuous lines. Spinal ganglia numbered.

- | | | | |
|---|-------------------------------|---|---------------------------------------|
| A | N. cruralis | K | R. cutaneus dorsi pedis lateralis |
| B | R. cutaneus femoris lateralis | L | R. cutaneus tibio-marginalis |
| C | R. profundus anterior | M | R. cutaneus cruris medialis inferior |
| D | N. ischiadicus (sciatic) | N | N. tibialis r. superficialis (sural) |
| E | R. articularis genu lateralis | O | R. cutaneus cruris medialis superior |
| F | R. articularis genu medialis | P | R. cutaneus cruris posterior |
| G | R. articularis genu et pedis | Q | R. cutaneus femoris medialis |
| H | R. cutaneus cruris lateralis | R | R. profundus posterior |
| I | N. peroneus | S | R. cutaneus femoris posterior |
| J | N. tibialis r. profundus | T | Cutaneous branch of 11th spinal nerve |

fate following experimental interference, it was concluded that these branches consist of proprioceptive fibers.

EXPERIMENTAL

A. Ventral root extirpation

Series A I. Removal of cord segment. As might be expected, survival among this group was poor. Besides locomotor disturbances, doubtless some visceral functions were affected by the operation. The condition of larvae which survived improved with time, becoming normal in most respects except locomotion. Observations of voluntary behavior and stimulation tests revealed all degrees of limb activity ranging from normal movements to complete paralysis. In the group classed as paralyzed, although movements could not be detected with the naked eye, sometimes slight jerking motions of the leg joints were seen under the microscope. These were wholly spontaneous and could not be elicited or increased by stimulation.

The number of animals in this series is shown in the following table.

CASES	DIED	TESTED FOR FUNCTION	MOVEMENT NORMAL	MOVEMENT IMPAIRED	PARALYZED
45	33	15	4	3	8
Sectioned for study			1	1	5

The following two cases may illustrate the type of limb innervation resulting from removal of the cord.

Case C4. Larva at operation in stage L3. The cord was transected at spinal segment 6 anteriorly, and segment 13 posteriorly, and the intervening piece was removed. Five days later the tadpole was placed in a weak thyroxin solution to hasten growth of the legs.

Functional tests 35 days after the operation showed that tactile stimulation of the feet and shanks elicited no response, but the thighs were sensitive to touch. With the unaided eye no movement of the legs or feet could be seen, but under magnification a slight spontaneous twitching was sometimes detected. A lateral flexure at the base of the tail had developed, otherwise the animal appeared normal.

The condition of the cord and sciatic plexus is illustrated in figure 2. The cut ends of the cord had healed over without regeneration. Both legs have almost identical innervation. With but one exception, all mixed and cutaneous branches are present. The cutaneous nerves are as large as those of a normal animal of the same age, and the skin is abundantly supplied by branches. The exception just referred to is

the absence of the femoris medialis branch to the skin (Q fig. 2). Probably in some way related to this lack is the unusual size and extent of the r. cutaneus femoris posterior which here occupies the normal territory of the missing branch. The general completeness of the sensory innervation, however, is well illustrated by the presence of even the very fine proprioceptive branches to the knee and ankle joints (E, F, G, fig. 2).

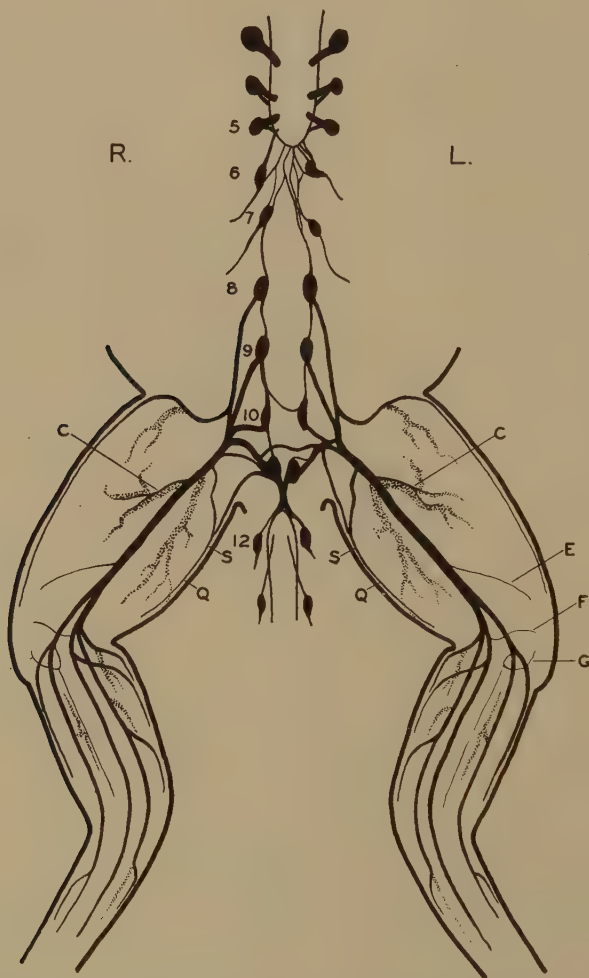


Fig. 2 Diagrammatic reconstruction of innervation in case C4. Stippling indicates position of nerve branches which have failed to develop. See text for further explanation.

In each leg there is just one nerve branch entering muscle tissue. It occupies the relative position of the profundus anterior branch which supplies the extensor muscles of the thigh (C fig. 2). In the left

leg it contains only twenty-five fibers, and in the right fifty. These branches end on striated muscle fibers, but because they are so few, it is impossible to determine whether they follow the forkings of the normal profundus anterior. All other muscles are entirely devoid of nerve fibers.

It is possible that a few V-fibers from the cut ends of the cord may have found their way into the plexus by following back along the dorsal roots which have grown forward to the stump of the cord. Thus it may be that the two small muscular branches in these legs consist not of D-fibers, but of motor V-fibers. This would account for the slight twitching movements observed. The appearance of all fibers contained in both muscle and cutaneous branches is entirely normal.

Case C8. Larva at operation in stage L4. The cord was cut at the seventh spinal segment and pulled out from the posterior portion of the spinal canal. Ganglia were left intact.

Later tests showed that general body movements could not be evoked by stimulation of the legs except on a small area of the left thigh. No leg movements could be elicited or occurred spontaneously.

The animal was killed and fixed 35 days after the operation at stage L13.

Histological examination revealed that only fine nerve branches extended craniad from ganglia, forming a network between them (fig. 3). Some strands reached the stump of the cord. Even though some V-fibers from the cord could follow these dorsal roots to the ganglia and finally reach the legs, their total number was very small.

The pattern of cutaneous branches within the legs is almost normal. However, for unknown reasons, all the nerves are smaller than corresponding nerves of unoperated animals. In the thigh of both legs the cutaneus femoris posterior and cutaneus femoris medialis branches (Q, S fig. 3) are wanting. Besides this lack it is seen that in both legs all the fibers of the suralis nerve enter the skin with the r. cutaneus cruralis medialis inferior (M fig. 3) thus omitting the mixed terminal portion extending into the foot (N).

There are no muscular branches nor nerve fibers in the muscles of the left leg. In the right leg, a small twig of only fifteen fibers leaves the sciatic at the normal point of origin for the profundus posterior (R fig. 3). These fibers are distributed to the muscles adductor magnus and adductor longus. Aside from this, the only other muscle innervation in the right leg is a single large fiber which arborizes and terminates in the belly of the gastrocnemius (U fig. 3). This single fiber can be followed back to the hip through subcutaneous tissue to the point

where it left the sciatic nerve. Because of its unusually large diameter it can be traced up the spinal nerve and past the ganglion to its origin in the end of the cord. Thus at least one of the fibers which end in muscle tissue can be shown definitely to be of ventral root origin.

The two cases described above illustrate the development of an almost purely cutaneous innervation in the absence of ventral root

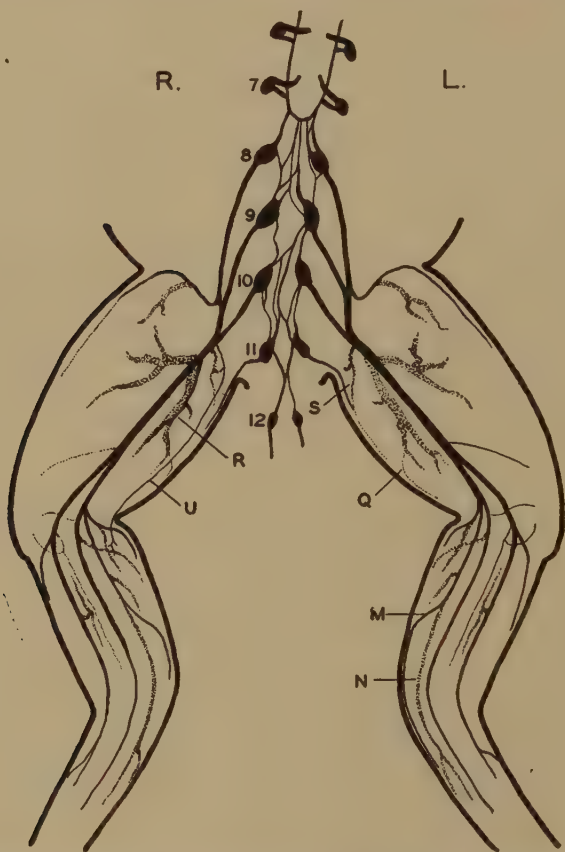


Fig. 3 Diagrammatic reconstruction of innervation in case C8. Stippling indicates position of nerve branches which failed to develop. See text for further explanation.

fibers. Of the remaining three larvae of this series studied in section, one had no ventral roots and no muscular nerves in the limbs, while the other two showed partial reestablishment of one or two ventral roots. In these later cases small nerves were present at the site of some of the normal motor branches of the limb resulting in partial innervation of muscles. Apparently this muscle innervation was insufficient to produce more than slight twitching movements. A distinct

relationship was observed to exist between the degree of regeneration of ventral roots and the number and size of muscular nerves found in the limb.

Various measures had been taken to prevent this regeneration of V-fibers into the limb. Among these were attempts to block the spinal canal by replacing the excised segment of cord by grafts of different tissues, such as muscle or lens, or by damming with a membrane of gall bladder. However none of these measures had been wholly effective, since about half of the surviving animals showed typical, though sometimes weakened, leg and foot movements.

B. Dorsal root extirpation

Series B I. Removal of spinal ganglia only. All but one of this series of animals were operated upon before the limbs were functional and hence the completeness of the operation could not be immediately tested. Development of the limbs after the operation proceeded normally. By stages L11 and L12 tests were made for sensitivity. These showed that half of the animals behaved in a normal fashion, even a light touch being sufficient to cause the tadpole to swim away. About one-fourth would respond only to stroking with a needle or pinching with forceps. In general the distal part of the leg was less sensitive than the proximal. In fewer than one-fourth of all tested animals the limbs were wholly insensitive, or would respond only after excessive stimulation which might have moved the whole leg sufficiently to have stimulated sensory nerves in other parts of the body. Even in these cases of completely insensitive limbs, normal motor limb function was observed, a fact already pointed out by Weiss ('41a).

Larvae from each of these groups were sectioned and stained for study. Invariably animals whose limbs had previously been shown by stimulation tests to be insensitive were confirmed to be without ganglia and D-fibers.

The following table gives the number of animals in each category.

CASES	DIED	TESTED FOR FUNCTION	SENSATION NORMAL	SENSATION IMPAIRED	INSENSITIVE
77	18	44	22	12	10
Sectioned			3	2	7

Cases best representing the results of this operation are described in detail below.

Case D19. Larva at operation in stage L4. Ganglia 8 to 11, inclusive, were removed from the left side.

Twenty-three days later the limb was capable of normal function, although it was used less than that on the control side. Tests showed it to be totally insensitive. It was somewhat smaller than the right leg.

The larva was fixed at stage L13.

The diagrammatic reconstruction (fig. 4) shows that the ganglia had been completely removed and that spinal nerves 8, 9, 10 and 11 of the left side have only ventral roots.



Fig. 4 Diagrammatic reconstruction of innervation in case D19. Stippling indicates position of nerve branches which failed to develop. See text for further explanation.

Although all muscles of the limb are supplied with a normal quota of fibers and most of the main muscular nerves are typically situated, certain striking abnormalities are seen in the innervation of the shank. These are chiefly associated with the absence of most of the peroneus divisions, only one of its branches, the cutaneus cruris lateralis, being represented. The distal part of the suralis is also missing. Since both of these normally supply motor and sensory fibers to the foot, the

tibialis remains as the sole source of nerves to that part of the limb. The dorsi-flexor muscles of the ankle joint, which have been left without innervation by the absence of the peroneus, now receive fibers from the tibialis by way of a new nerve (U fig. 4) that passes to them through a foramen in the tibiofibula normally occupied only by a blood vessel.

It is questionable whether this abnormality should be ascribed to the operation or had not better be considered a chance aberration, since among the twelve other larvae similarly treated and studied in serial section, no comparable alteration was observed.

Besides the muscular nerves, however, branches were seen extending to the skin. Although these resembled typical cutaneous branches in their place of origin from the mixed nerves and in their position in the skin, they differed markedly in their small size and in the fine caliber of their fibers. The faintness of the silver impregnation of these fine fibers made it impossible to follow them to their terminations, in fact, in most cases the whole nerve could not be traced very far after it entered the cutis.

Case D11. Larva at operation in stage L4. All ganglia from 7 to 12 were removed from the right side. The tenth ventral root was severed in the operation. At stage L12 the right leg was considerably smaller than the left, being about four-fifths as long. It was not sensitive to stroking or pinching with forceps although it exhibited all normal movements.

The animal was killed and fixed 28 days after the operation.

There was a total lack of ganglia on all the right spinal nerves supplying the limb. However, a branch (V fig. 5) coming from the eleventh mixed nerve on the left and passing dorsal to the cloaca breaks up into four parts and enters the eleventh spinal nerve of the right leg. Two of these branches go directly into the skin of the thigh accompanying the cutaneous branch of the eleventh (T) and joining the r. cutaneus femoris posterior (S), the other two join the eleventh nerve above the plexus.

The muscular innervation pattern is normal, although the branches measure somewhat less in diameter than those of the unoperated left side. This may be due to the smaller size of the right limb, an interpretation suggested by the condition existing in regenerating limbs of *Triturus* where Litwiller ('37) finds a definite relationship between the number of fibers and the mass of the limb.

The leg is abundantly supplied with cutaneous fibers in the region of the thigh. Most of these can be traced to the normally small cu-

taneous branch from the eleventh nerve (T) which in this case is considerably increased by contributions from the left side through the cross-branch. A second comparatively large branch to the skin of the thigh is the r. cutaneus femoris posterior (S) which is also probably augmented by fibers from the left side by way of the plexus and sciatic trunk. With but one exception, the remaining cutaneous nerves of the



Fig. 5 Diagrammatic reconstruction of innervation in case D11. Stippling indicates position of nerve branches which failed to develop. See text for further explanation.

leg are represented. None of the proprioceptive branches could be found. The fibers which compose the cutaneous branches are of two varieties, fine ones such as those described for case D19, and large ones indistinguishable from typical cutaneous fibers.

Case D57. Larva at time of operation in stage L1. An attempt was made to remove ganglia 7 to 11 from the left side. Forty-one days later when the tadpole had reached stage L6, the eighth, ninth and tenth ganglia were removed from the right side. The eleventh was torn in

operation and its removal was not certain. Tests a month later showed both legs slightly sensitive to pinching. They took part in normal swimming movements.

At stage L13 the larva was killed and fixed.

Sectional study showed that ganglion 8 is present on the left, and numerous ganglion cells are at the eleven locus on the right. The two legs appear similar in their innervation pattern. In both, the muscular nerves are typical in arrangement and size and the muscles are well innervated. In both, also, the major cutaneous branches, though small, are present and composed of several large fibers accompanied by a group of fine ones. One or two of the smaller nerves are wanting in each leg and the skin is poorly supplied with fibers. All the branches bear their normal relations to mixed nerves and terminal tissue.

Series B II. Removal of spinal ganglia followed by limb amputation.

In order to test more critically the results obtained in series B I, the same operation was carried out on older larvae, thus eliminating largely the danger of incomplete ganglionectomy. To obtain limb buds that would be comparable to those of an animal at a much earlier stage, the old buds were cut off close to the body wall allowing a new one to reorganize from the remaining limb field material. These secondary limbs were in every way normal in appearance, and only slightly delayed in development.

Four tadpoles were operated in this series. Of these one died. The remaining three were sectioned and studied. Since both limbs of each larva were used six cases were actually observed.

Case D51. Larva at operation in stage L5. Ganglia 7 to 10, inclusive, were removed bilaterally. Both limbs were amputated close to the body wall.

When tested 25 days later, there was no response to stroking or pinching in either leg. Both appeared normal and functioned in swimming.

Figure 6 shows the resulting distribution of nerves. The operation had been complete except for two ganglion cells which remained on the left tenth spinal nerve. The nerve branches to muscles of the right leg are normal and complete. The same is true for the left with the exception that the distal part of the mixed suralis nerve (N) to the foot is missing. The cutaneous innervation, on the other hand, is very deficient both in number of branches and in their size. Of the thirteen cutaneous and proprioceptive branches considered in these studies eight were found represented in the right leg and six in the left. One of the fine proprioceptive branches of the left leg (W fig. 6) instead of ending

in the connective tissue of the joint, passes into the distal end of the cruralis muscle. All the cutaneous branches present, despite their small size, are normally situated and all reach the skin.

Series B III. Removal of spinal ganglia followed by transection of sciatic plexus.

Boeke ('17) showed that in the tongue of the hedge-hog sensory nerves were capable of forming morphological connections with

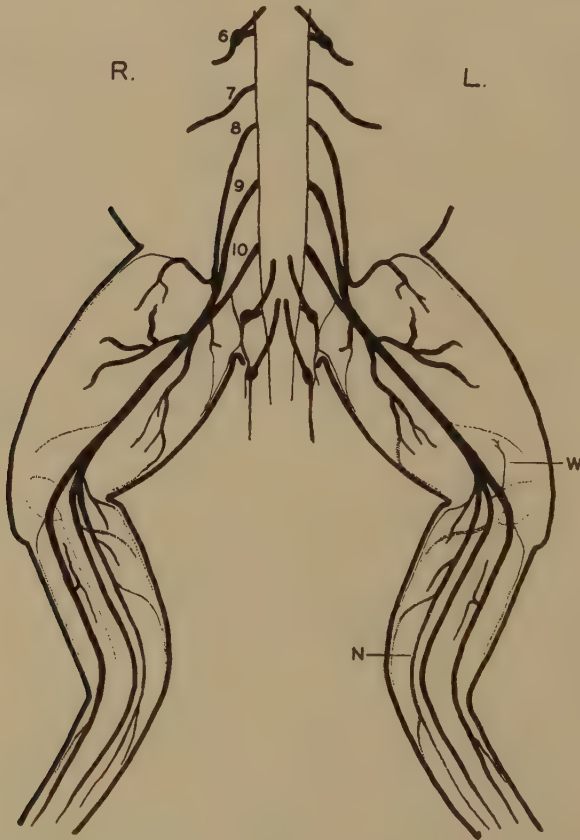


Fig. 6 Diagrammatic reconstruction of innervation in case D51. Stippling indicates position of nerve branches which failed to develop. See text for further explanation.

muscles. Later Weiss (34b) demonstrated in the toad that by electrical stimulation of a sensory nerve forced to grow into a muscle, the latter could be made to contract. In these experiments the cut end of a sensory nerve was either sutured onto the distal stump of a motor nerve or inserted directly into a denervated muscle. There was thus no alternative for the fibers but to enter a foreign terminal tissue.

The experiment of series B III was designed to determine whether, in the limb, regenerating nerves would discriminate between the old sensory and motor pathways when both were made available to them. For this purpose older larvae, in which the limb innervation had already been established, were used. Several combinations of ganglionectomy and plexus transection were made in these operations so that one limb would serve as a control for the other. In one group, "A" (fig. 7A) ganglia were removed bilaterally and the plexus transected on only one side. In a second group, "B", the ganglia of one side were removed and the plexus of both sides cut (fig. 7B).

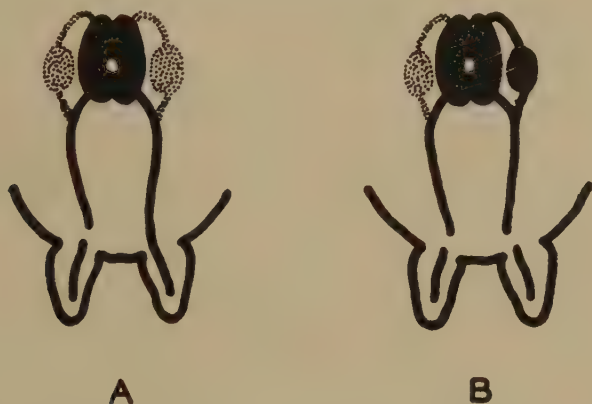


Fig. 7 Diagram of the operations performed on larvae of series B III. Ganglia and dorsal roots removed are shown stippled. See text for further explanation.

In all cases the legs remained anesthetic on the sides where ganglia had been removed.

The animals were fixed and sectioned after allowing time for regeneration of the nerves as indicated by return of motor function.

This series includes bilateral operations on ten animals. Two of these died. Five of the remaining were studied in serial sections.

An example from group A is given below.

Case TA3. Larva at operation in stage L10. Ganglia 8, 9, 10, and 11 were removed from both sides. Four days later the left plexus was transected.

After 18 days, when the tadpole was in stage L14, no response could be obtained from stimulation of either leg although normal voluntary movements were observed.

The animal was fixed at this stage and sectioned.

Histological examination revealed that the operation had been complete so that no D-fibers could have reached the legs. Yet in addition

to their normal muscle innervation, both legs show a nearly complete set of cutaneous branches. These are very small and consist of fibers much finer and more lightly impregnated than those of the motor branches. There are no large fibers accompanying the finer ones.

Despite the fact that the sciatic of the left leg had been cut 18 days previously, there appears no difference in the innervation pattern or of the relative size of muscular and cutaneous branches in the two legs.

In the connective tissue and fascia of the proximal portion of the thigh stray fibers are present, most of which terminate in muscle tissue.

An example from group B is the following.

Case TB2. Larva at operation in stage L11. Ganglia 8, 9, 10 and 11 were removed from the left side.

Ten days later the sciatic plexus on both sides was cut.

After 18 days normal movements and responses were observed in the right leg. The left leg would move when the right was stimulated, and it was used in swimming, but no response could be elicited by tactile stimulation on any part of its surface.

The larva was killed and fixed at stage L15.

Study of the sectioned animal shows no trace of left ganglia 8, 9, or 10 except for four ganglion cells at the locus of the eleventh. Since there were no cross branches from the right side, it is evident that no D-fibers could reach the left leg except those arising from the four cells on the eleventh spinal nerve.

The innervation of the right leg is normal in arrangement of its branches. The left leg also has a normal pattern but the cutaneous branches are very small and consist of fine fibers. The three proprioceptive branches to the knee were absent. Measurements of diameters of the nerve branches showed the muscular nerves of the two sides to be equal. On the left side the mixed nerves were generally a little smaller than on the right, while the cutaneous branches were less than one-third as large, the proportions varying with the different branches.

It appears from the results in both groups of this series that after transection the regenerating fibers do not grow out at random with respect to the former muscular and cutaneous pathways. The invariably reduced size of the cutaneous branches where no D-fibers were permitted to reenter the limb, indicates a strong selectivity of the V-fibers for muscular branches.

C. Controls

Degeneration. In order to ascertain whether both V- and D-fibers are present in the early stages of limb development and also to de-

termine the distribution of the two types of fibers in the normal limb nerve pattern, a series of degeneration experiments was carried out. These consisted in the removal of ganglia or the cutting of the ventral roots in the limb region of tadpoles at different stages of development. After allowing 2 or 3 days for degeneration of the distal portion of the fibers thus severed from their cell bodies (Williams, '30; Speidel, '35), the larvae were fixed and sectioned for histological examination.

Because of the difficulty encountered in removing ganglia of young larvae, only the ventral root transection was attempted at stages L1 and L2. In a tadpole in each of these stages the left ventral roots 8, 9, 10 and 11 were cut and 3 days later the animal was fixed. In both stages L1 and L2 the mass of fibers was found to be smaller at the base of the left limb bud than at the right. The same result was observed in two larvae of stage L4 that had been similarly treated. Although the number of animals tested is small, the results indicate that the fiber mass at the disposal of the early limb bud contains both dorsal and ventral root elements. This is in agreement with an observation previously reported (Taylor, '43) that fibers of the early limb innervation could be traced in histological preparations back to their sources in both the cord and the spinal ganglia.

The right limb ganglia were removed from an animal each of stages L6, L11 and L15. After again allowing time for degeneration they were fixed, sectioned and impregnated with silver. In each of these, motor branches were normal, while the cutaneous branches on the operated side were in process of degeneration. In the oldest larva, however, although all the fibers of large diameter had disappeared from the cutaneous branches a small number of the finest fibers were still present and showed no signs of degeneration.

These control experiments demonstrate that limb innervation includes, from the beginning, both D- and V-fibers; also that in normal development muscular branches contain only V-fibers, while cutaneous branches contain D-fibers and a few fine fibers of undetermined origin.

Ganglion implantation. To test further the selectivity of fibers growing into a limb with already developed nerve pattern, ganglia were implanted into a group of twenty tadpoles. These implantations were made after removal of the cord as in series A I. The ganglia were inserted either into the spinal canal or under the skin at the base of the limb bud. In none of the cases where implants had been placed under the skin could they be located later by looking through the transparent skin, and in no case where histological studies were made were ganglion cells or fibers seen. The site of the implant was marked only by scar tissue.

DISCUSSION

Selectivity of dorsal root fibers in development

That D-fibers establish selective connections with their normal (homologous) type of terminal tissue rather than with muscle (heterologous) tissue, is clearly indicated by the general lack of muscular branches in V-less limbs (series A I) while the cutaneous branches are, on the whole, normal in distribution and size.

All cutaneous branches present arise in the typical locations along the main trunks or divisions. The reason for the occasional absence of some of the smaller cutaneous branches is not clear. Since the deficiency was greatest in the right limb of case C8, which, for unknown cause, is weakly innervated, the number of fibers available may have something to do with the success of the establishment of a nerve branch. This, however, would not apply for the cutaneous femoris medialis branch which is absent in every animal of this series studied in sections (see below).

The lack of muscular nerves is apparently complete in case C8, while other cases show traces of muscle branches. The probability that all such traces which do appear are formed by V-fibers from motor cells is suggested by the fact that the number and size of these branches is directly proportional to the volume of V-fibers regenerated into the limb; in several cases fibers ending in muscle can actually be traced back to the cut end of the spinal cord. Furthermore, if dorsal root fibers were capable of penetrating muscle tissue and forming motor endings, it would be difficult to explain the lack of the majority of muscular branches as well as the small size of those few which do exist, in limbs which had received an abundant supply of D-fibers. If D-fibers can enter either skin or muscle with equal ease, one would expect V-less limbs to have an equal reduction in the size of both the muscular and cutaneous branches of the innervation pattern.

In normal development the essential features of the innervation pattern are laid down at a very early stage in morphogenesis of the limb (Taylor, '43). From this time on, the pattern is extended and fashioned by the growth of the skin, muscles and skeleton of the leg. Thus a normal nerve pattern will develop if all the normal branches have established connections with their terminal tissues at the proper time. Failure of one branch to form such a connection would result in the omission of that particular branch from the final pattern without materially affecting the normal course of the rest.

This interpretation accounts for most of the irregularities encountered in animals of series A I. The general lack of nerve branches

to the muscles indicates the failure of D-fibers to attach to muscle primordia. The few V-fibers which may be present assume, in every case, the position of a normal muscular branch, and like all other branches, extend to their typical terminations. The comparatively few cutaneous branches which are lacking must likewise have never been formed, due to lack of sufficient pioneer fibers or their failure to establish an end connection.

In a reference to this work made during the early stages of the investigation, Weiss ('41b) has stated that the selectivity is not strict. Since then, however, further work has shown that, at least in the case of D-fibers, there is definite specificity for homologous terminal connections.

Selectivity of ventral root fibers in development

The D-less innervation patterns resulting from the operations of series B I, B II and B III exhibit two general characteristics: (A) The completeness of that portion of the pattern which supplies the muscles, and, (B) The presence of a typical cutaneous pattern which is often incomplete and always composed of very fine branches.

As for the former, the presence of nerves in all the muscles doubtless indicates simply that V-fibers were at hand when end connections could be made with the primordial muscle cells. Dorsal and ventral root components are thus alike in the respect that each is capable of producing its own typical distribution pattern in the absence of the other. Deficiencies of muscular innervation, such as described by Yntema ('43) following excision of embryonic neural crest, were not seen in any of the cases of removal of spinal ganglia.

The fact that, in addition to the muscular nerve pattern, a typical cutaneous nerve pattern also develops, raises the problem of the origin of these cutaneous fibers. In all the animals of series B I there exists the possibility that a few D-fibers may have entered the limb through branches seen to connect a near-by ganglion, either on the same or opposite side, with its sciatic plexus. To circumvent this difficulty the experiments performed in series B II were devised. With the older larvae used in this series, the ganglia could be completely removed with certainty, while a limb bud without previously formed nerve courses was obtained by amputating the old one and allowing a new bud to be reorganized. In animals so treated and shown by careful histological examination to have received no D-fibers there still appear those typically situated cutaneous branches.

The fact that these larvae showed no response to stimulation of the limbs indicates that the fibers in the cutaneous nerves are not sensory. Further evidence of this fact is to be seen in the character of the fibers themselves. They are extremely small and impregnate poorly with silver (fig. 8), thus showing little resemblance to the large D-fibers of a normal cutaneous nerve. This contrast is striking in cases of incomplete ganglionectomy where the fine fibers are accompanied by

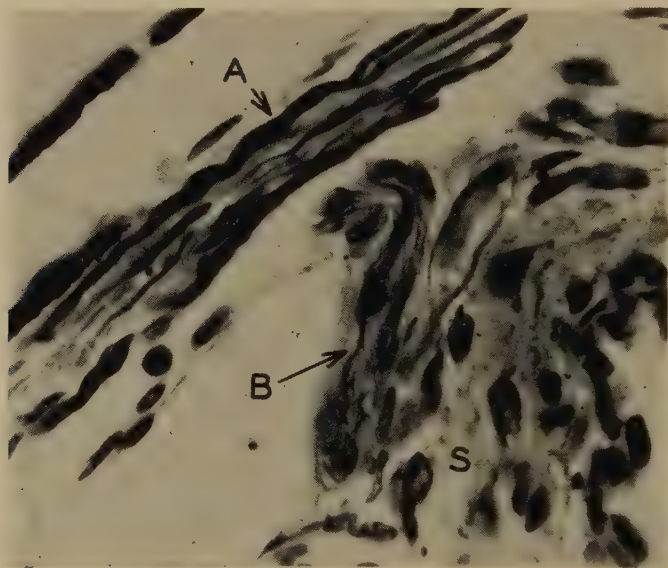


Fig. 8 Photomicrograph showing contrast between size of fibers in muscular branches A and small cutaneous branches B in limbs of larvae from which spinal ganglia had been removed. S, skin. Bodian. $\times 700$.

a few cutaneous fibers of normal appearance. It is evident that cutaneous branches can be formed by fibers other than those from spinal ganglia.

It is possible that these cutaneous branches are composed of V-fibers which, because of their heterologous terminations, have failed to grow in diameter. If so, then V-fibers would differ from D-fibers in their lack of specificity for homologous termination. Even in normal larvae some of the fine fibers contained in cutaneous branches were shown by the degeneration tests to persist after ganglionectomy. Presumably these originate from the same source as the fibers which form the cutaneous branches in experimental D-less limbs. The possibility that in both cases these may be V-fibers rests on the assumption that V-fibers

will not grow to normal size if they terminate in heterologous tissue; an assumption with little supporting evidence.

A second possibility is that these are postganglionic sympathetic fibers which normally innervate the numerous glands of the skin. This is suggested by the fact that in the histological preparations fibers of similar diameter and staining properties could be traced from the ganglia of the sympathetic trunk into the spinal nerves supplying the limbs. Since they could be traced for only a short distance peripherally their identity with fibers of the cutaneous branches could not be demonstrated. However, the fact that similar fine fibers were absent from the muscular branches of V-less limbs falls in line with the reported observation that skeletal muscles do not receive sympathetic innervation (Hinsey, '27) (Wilkinson, '29).

If the fine cutaneous fibers of the D-less limbs have their origin in sympathetic ganglia rather than in the spinal cord, then quite obviously V-fibers would be as strict as D-fibers in their selectivity of terminations in homologous tissue. Unfortunately the evidence regarding the actual source of these fibers is insufficient to allow a final decision.

Nature of the selective action

Previous work has revealed little regarding the mechanism by which the different types of invading nerve fibers achieve connections with their appropriate terminal tissues. Weiss ('34a) has considered the possibility of explaining this phenomenon by the timing of the arrival of the two types of fibers in synchrony with periods of acceptance of the homologous tissues. Such an explanation is, however, contradicted in the case of the frog limb bud by our degeneration experiments, which reveal the presence of both V- and D-fibers in the sciatic plexus of larvae as early as stage L1.

The determination of final connections may conceivably occur in either of two ways. It may be either a primary elective process, involving the laying down of branches which are from the beginning either sensory or motor. Or it may take place after an initial indiscriminate establishment of all branches by both types of fibers, by a secondary selective elimination of those inappropriately connected.

Careful examination of the V-less limbs showed absence of any trace of nerves which might formerly have extended to the uninervated muscles. Thus in the case of D-fibers, at least, there is some evidence that terminal connections result from an early elective process rather than one of secondary elimination. This is probably true also of

V-fibers if we may assume that they do not form the cutaneous branches of D-less limbs.

Ample evidence has been presented (Weiss, '34a, '41b; Weiss and Taylor, '44) to show that orientation of advancing fiber tips is achieved by contact of the nerve fiber with oriented structures within the substrate. The significance of the mesostroma in directing the course of nerve fibers which first invade the limb bud has been previously pointed out (Taylor, '43). It is possible that the part played by the stroma is neutral with respect to the different fiber components, and that differential selectivity is not expressed until the fiber tips actually contact the cells of the presumptive terminal tissue. On the other hand, local biochemical differentiation occurring within the limb mesenchyme may affect the adjacent stromal strands in such a way as to cause them to act as specific conductors of a particular type of nerve fiber (Weiss, '41). Thus selection of the pioneer fibers would be in terms of preneural pathways rather than of the terminal tissues themselves. Whereas both the concept of selection of terminal tissue and that of the selection of a stromal pathway would provide for the proper terminal connections of individual fibers, only the latter would furnish a plausible explanation for the formation of discrete nerve branches to the various types of terminal tissues.

Following the selection of preneural pathways by the pioneer fibers there occurs a selection of established neural pathways by fibers which grow out later. Evidence for the latter type of selection may be seen in both normal and experimental animals. At no time during normal development are terminal nerve branches found to contain both types of fibers, as would be the case if out-growth were random with respect to neural pathways. Among the experimental larvae the extremely small size of the few muscular branches in the V-less limbs indicates this form of selection. These well established muscular branches remained permanently very small in spite of the fact that new D-fibers were continually growing into the limb. Obviously D-fibers had passed them by. The process by which a young fiber selectively follows an older fiber, or group of fibers of its own type has been designated as "selective fasciculation" (Weiss, '41b). In this way the arrival of secondary fibers in homologous terminal tissue is assured from the start.

Significant irregularities in the nerve pattern

On the whole, the innervation pattern of frog limbs is quite constant. Irregularities of the major branches were not found in any of the

normal larvae. In the experimental animals, with few exceptions, the abnormalities encountered were all omissions of branches attributable to deficiencies in the fiber source. The origin and distribution of existing branches were always typical and, excepting for an occasional stray fiber, no aberrant extra branches were observed.

Piatt ('42) has described the innervation of *Amblystoma* forelimbs when nerve fiber invasion had been delayed until after differentiation of the limb elements. Although he finds some aberrations in the resulting nerve patterns, he stresses the basic similarity of these patterns to the typical limb nerve distribution. However, the irregularities which Piatt observed contrast with the stereotyped configuration of the nerve branches in the experimental cases here presented. The factors involved in Piatt's cases and in undelayed innervation are quite different. In the former, pioneer nerve fibers must "explore" their way between differentiated elements of the limb, while in the latter the active period of the pioneer fibers is largely ended before visible differentiation of limb tissues (Taylor, '43).

Although in experimental limbs the cutaneous nerves arise and approach the skin in a typical fashion, a few instances of increased extent of ramification within the skin deserve attention. These occur when normally adjacent skin branches had failed to develop. Thus, for example, in case C4 the r. cutaneus femoris posterior (S fig. 2) is extended into the region usually innervated by r. cutaneus femoris medialis (Q) which is here missing. Also in case D11 where a connecting branch from the left side (V fig. 5) had furnished fibers to the right r. cutaneus femoris posterior (S). Thus the latter is several times its normal size and the distribution of its branches in the skin is correspondingly increased. It supplies much of the area usually innervated by the r. cutaneus femoris lateralis (B), which is here much reduced, as well as that of the r. cutaneus femoris medialis (Q). The r. cutaneus cruris medialis inferior of case C8 (M fig. 3) is still another example.

Speidel ('41) has observed the formation of new sprouts on intact nerve fibers near a denervated area of skin. Although he suggests that this response of the nerve fibers is due to stimulation by the denervated zone, it can be equally well explained in terms of the removal of an inhibiting "saturation factor". Similar invasion of denervated areas by collateral fibers from surrounding nerves has been demonstrated histologically in the adult rabbit by Weddell ('42), and is well known clinically in man.

In addition to the excessive terminal branching some of the experimental cases cited above show also an increase in size of the whole cutaneous nerve. This offers evidence that the periphery exerts an influence in determining the number of its innervating fibers.

In six of the experimental cases a nerve branch, crossing over the midline, connects the plexuses of the two sides. The same type of crossed innervation was observed by Hamburger ('28, '29) and was considered by him as evidence of the attraction of nerve fibers by limb tissue. That the deviation cannot be due to a demand of the limb for a particular fiber type is evidenced by the fact that it occurs also in cases where both limbs lack one fiber type, i. e., C4 with both limbs V-less.

This particular case, (C4), furthermore, serves to contradict any attraction from a distance in the formation of such cross-branches; for, since both limbs exhibit an equal innervation deficiency, they would presumably emit an equal attractive force, in which case the fibers of either plexus would be more strongly attracted to the limb of its own side than to the more distant opposite limb.

These cross-branches, moreover, can be readily accounted for without recourse to attractive forces. Significantly, the position of the connecting branch was, in every case, at the level of the eleventh ganglion between the notochord and cloaca. This suggests some preexisting structural bridge. Examination of normal larvae, in fact, reveals that a small branch from the tenth spinal nerve of either side passes medially along the dorsal surface of the cloaca, approaches its mate from the opposite side and anastomoses with it. It is apparently this branch which can serve as pathway from one side to the other. Although such a crossing over of fibers may occur in normal animals, it remains on a small scale, possibly because the terminal tissues are found "saturated" with ipsilateral nerves. If, on the other hand, such a fiber, crossing over by chance, should find the invaded limb tissue uninnervated, a terminal connection could readily be made and the fiber, thus established, would serve to conduct other fibers across the midline by selective fasciculation.

Mention has been already made of the absence of the r. cutaneous femoris medialis in the V-less limbs of series A I. Since this abnormality was found in every case studied, it would appear to be related in some way to the deficiency of V-fibers. A possible explanation is suggested in the development of this branch. When first detectable, in stage L5, it consists of a few fibers extending from a cone of pioneer fibers which later will become a large muscular nerve (*profundus posterior*) and which has penetrated through a mass of myoblasts. It

is conceivable that the cutaneous fibers in the primordium of this branch are dependent upon the accompanying pioneer muscular fibers to lay down for them a structural pathway through the myoblast mass which otherwise would serve as a barrier to their reaching the dermal tissue. Thus in limbs lacking muscular fibers, this barrier of heterologous tissue might be sufficient to prevent the formation of a cutaneous branch at this point.

A reciprocal relationship seems to be illustrated by the frequent absence of the distal part of the suralis branch after either type of experiment (cases C8, D51, and D19). The distal portion of this nerve consists, at the time of the operation, of only a very few pioneer fibers. Operative removal of either component may reduce the vigor of growth of the branch sufficiently to prevent its further advance.

It is thus apparent that, whereas in the main each component is independently able to establish its own typical pattern, instances of interdependence between the components do exist. These abnormalities serve also to emphasize a point made earlier, i.e., that the factors operating in the establishment and growth of one branch may differ from those of any other branch. This is true because of the part played by the environing local tissues whose structural configuration, chemical differentiation and growth changes are so important in the determination of the course of outgrowth and final position of every nerve branch.

There remains little doubt that invading nerve fibers exhibit a selective action which leads to homologous terminations. Although some insight into this process of selection has been afforded by the study of the development of each component alone, our knowledge of it is still meager and much work remains to be done before a clear understanding of its mechanism can be gained.

SUMMARY

The relationship of nerve fibers from the dorsal and ventral roots to their terminal tissues in the developing limb bud of the frog was investigated by experiments in which the spinal ganglia, or portions of the spinal cord were removed at an early larval stage.

In the absence of ventral root fibers, no muscular nerves were established, while the remaining dorsal root fibers produced a typical pattern of cutaneous nerves.

In the absence of dorsal root fibers, typical muscular nerves were developed. In addition, however, many cutaneous nerves were also present, although they were small and composed of very fine fibers. The

possibility that these cutaneous nerves are composed of sympathetic and not ventral root fibers is suggested.

Termination of nerves in homologous tissue is achieved by a selective action of the nerve fibers.

The selectivity of pioneer fibers is probably one of preneural pathways rather than of the terminal tissues themselves. Fibers growing out later exhibit selectivity relative to established neural pathways.

The significance of certain irregularities in the nerve patterns is discussed.

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